PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Philip E. Thorpe and Sophia Ran

Serial No.: 09/998,833

Filed: November 30, 2001

For: Combined Cancer Treatment Methods Using Antibodies to Aminophospholipids (As Amended)

Group Art Unit: 1642

Examiner: Fetterolf, B.

Atty. Dkt. No.: 4001.002299

CERTIFICATE OF MAILING
37 C.F.R. § 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450:

May 16, 2006
Date

Shelley P.M. Fussey

SUBMISSION OF EARLIER DECLARATION INTO CURRENT APPLICATION; COPY OF DECLARATION OF ADRIAN L. HARRIS UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicants respectfully submit the attached Declaration for formal consideration in the above-referenced application. Original versions of the attached Declaration and accompanying exhibits were submitted in U.S. Application Serial No. 08/457,869, now U.S. Patent No. 6,051,230, to address one of the same issues raised in the present specification.

Respectfully submitted
PEREGRINE PHARMACEUTICALS, INC.
Customer No. 000052101

Shelley P.M. Fussey, Ph.D.
Reg. No. 39,458
Agent for Applicants

5353 W. Alabama, Suite 306
Houston, Texas, 77056
(713) 439-0108

Date: May 16, 2006

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Thorpe & Burrows

Serial No.: 08/205,330

Filed: March 2, 1994

For: METHODS AND COMPOSITIONS FOR TARGETING THE VASCULATURE OF SOLID TUMORS

Examiner: Chapman Kelley, L.

Group Art Unit: 1208

Atty. Dkt: UTSD:393/PAR

CERTIFICATE OF HAND DELIVERY

I hereby certify that this correspondence is being hand delivered to Examiner Chapman Kelley, Group Art Unit 1211, Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

6/17/97
Date

Jacqueline Nitschmann
Signature

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF ADRIAN L. HARRIS
UNDER 37 C.F.R. § 1.132

I, ADRIAN L. HARRIS, HEREBY DECLARE AS FOLLOWS:

1. I hold the positions of Professor of Clinical Oncology and Director of the Imperial Cancer Research Fund Medical Oncology Unit at the University of Oxford and the Oxford Radcliffe Hospital, Oxford, U.K. I have held these positions for about 8 years. Immediately prior to my present employment, I held the position of Professor of Clinical Oncology at the University of Newcastle Upon Tyne, U.K. My research expertise is primarily in the fields of medical oncology, tumour angiogenesis, breast cancer research and treatment, and new drug trials. A copy of my *Curriculum Vitae* is attached hereto as **Exhibit A**.

2. I reside in the United Kingdom and am a British subject. I do not have a financial interest in matters related to the above-referenced patent application. However, I am being compensated for my time in preparing the present Declaration.

3. I have reviewed the specification, drawings and claims of the referenced patent application. I have also reviewed the scientific articles subsequently published by the inventors and colleagues in peer-reviewed journals: Burrows *et al.* (*Cancer Research,* 52:5954-5962, 1992); Burrows and Thorpe (*Proc. Natl. Acad. Sci., U.S.A.,* 90:8996-9000, 1993); and Huang *et al.* (*Science,* 275:547-550, 1997); copies of which are attached hereto as **Exhibits B, C** and **D.**

4. I have further reviewed the latest Official Action issued by the U.S. Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application. I have also studied the cited scientific articles: Hagemeier *et al.* (*Int. J. Cancer,* 38:481-488, 1986); Thorpe *et al.* (16th L.H. Gray Conference 1990); and Thorpe *et al.* (*Int. Symp. on Angio.*, 1991). It is my understanding that the Examiner believes the foregoing Hagemeier, Thorpe and Thorpe references to each be relevant to the claims of the above-referenced patent application so that they separately, or in combination, destroy the novelty or render obvious the claims of this application.

5. I understand the present invention, as disclosed and claimed in the referenced patent application, to concern methods and compositions for tumour imaging and for treating solid tumours based upon the use of antibody-selected agent constructs that localise to the intratumoral vasculature within a solid tumour. The antibody targeting regions of the conjugates bind to markers that are expressed, accessible to binding or otherwise localised on the endothelial cell surface of the established, intratumoral blood vessels within a vascularized tumour mass.

6. I disagree with the assessment that the Hagemeier reference and the Thorpe abstracts are relevant to the invention disclosed and claimed in the referenced patent application. My disagreement is based upon the fact that the studies described in the Hagemeier and Thorpe articles evidence an intent to target an antibody or immunotoxin solely to the budding capillaries within a vascularized tumour and, as such, represent the concept of anti-angiogenic therapy. I am particularly providing this declaration to explain that anti-angiogenic approaches to inhibit the proliferation of budding vessels within a tumour are completely different from, and do not suggest, methods of targeting the established, intratumoral vasculature of a solid tumour, which is the subject of this invention. It is also my objective to explain that, from studying the Hagemeier and Thorpe references, a skilled scientist could not reasonably expect to achieve meaningful tumour vasculature destruction or tumour regression by following the methods described in these references or variations thereof.

Hagemeier Concerns the Intent to Target Budding Vessels

7. The approach described by Hagemeier involves using antibodies intended to be reactive with endothelial cells of budding vessels in tumours for the purpose of inhibiting angiogenesis, *i.e.*, for inhibiting the development of new blood vessels in tumours. This reference does not suggest targeting the established vasculature within a tumour mass. Following the teachings in the Hagemeier article, I would not reasonably expect to achieve significant intratumoral antibody localisation or to be able to induce meaningful tumour necrosis or tumour regression following the approaches described by these authors.

8. To support the above conclusion, I draw attention to the introductory section of the Hagemeier article, which explains that their studies were initiated to raise antibodies against endothelial cell types involved in the *de novo* formation of capillary vessels. The EN 7/44 antibody that resulted from the Hagemeier studies is described as reacting "only with endothelial cells at the tip of a budding capillary vessel" (Hagemeier at page 481, column 2, lines 1-2). I

further draw attention to the statements made in the Hagemeier reference concerning the intention to develop antibodies capable of interfering in the complex sequence of events in angiogenesis, and again refer to the introductory sections at page 481, column 1 and the discussion at page 487, column 2. I do not see this reference as suggesting the generation of antibodies for use in tumour destruction, but understand the intent of these authors to investigate angiogenesis and the potential of anti-angiogenic methods to prevent further metastasis or enlargement of existing tumours. The authors specifically state that "antibodies appear to be useful in dissecting the complex sequence of cellular and molecular events of angiogenesis in normal, inflammatory and neoplastic tissues and have an interesting potential for the diagnosis of pre-malignant and malignant lesions in histological sections" (concluding paragraph of Hagemeier).

9. I also question the persuasive value of the data in the Hagemeier reference, even as it pertains to the EN 7/44 antibody and the apparent proposal to use this antibody in anti-angiogenic methods. This reference describes the target antigen of the EN 7/44 antibody as being found in the cytoplasm of human placental and umbilical vein endothelial cells (HUVEC). However, the authors also state that this antigen is found on the surface of tumour endothelium. The evident contradiction between the cytoplasmic location of the antigen within HUVEC and the apparent surface location of the antigen within tumour endothelium is not adequately explained by the data presented in this reference. The data that is reported to demonstrate the surface expression of the EN 7/44 antigen on tumour endothelial cells is described in Figure 2 of the Hagemeier article (page 483). However, the data presented in Figure 2 lacks persuasive value for many reasons, including: the lack of a scale on the axes; the lack of effective controls in this study; the failure to show that the cells in question are indeed endothelial cells; and the marginal advance of the EN 7/44 staining above background levels. In fact, rather than demonstrating that the EN 7/44 antigen is expressed on the surface of tumour endothelial cells, I would interpret this data as providing little evidence in support of surface expression and even tending to indicate that the EN 7/44 antigen is an intracellular antigen. Targeting of such an

intracellular antigen could, therefore, not be achieved by administering an antibody or antibody-based conjugate into the bloodstream of an animal.

The Thorpe Abstracts Concern Targeting Only Proliferating Endothelial Cells

10. Each of the Thorpe abstracts concern the PB1 antibody and attempts to use this antibody to selectively kill proliferating microvascular endothelial cells. The Thorpe abstracts do not suggest targeting the established, intratumoral vasculature within a solid tumour, but again represent an attempt at anti-angiogenic therapy. I do not believe that the data in the Thorpe abstracts would lead a skilled scientist to reasonably expect to be able to induce meaningful tumour necrosis or to cause tumour regression upon administering an immunotoxin based upon the PB1 antibody.

11. My assessment of the Thorpe abstracts as lacking relevance to this invention is again based upon the fundamental difference in approach between anti-angiogenic therapy, as represented by the Thorpe abstracts, and anti-vascular targeting and destruction, as provided by the present invention. One of the Thorpe abstracts indicates that injection of a PB1-based immunotoxin into a sponge implanted in an experimental animal delayed, although did not prevent, vascularization of the implanted sponge. This result is of the type that may be expected from an anti-angiogenic therapy, *i.e.*, the administered agent may be expected to inhibit the growth of new blood vessels. However, there is no indication or expectation that established, blood transporting vessels could be targeted or destroyed upon administration of PB1 or a similar type of antibody. In fact, I would not expect that agents targeted to the proliferating microvasculature, as described in the Thorpe abstracts, would produce an anti-tumour effect as these types of vessels are not critical to tumour survival. Targeting such proliferating microvasculature could only be reasonably expected to be capable of preventing even further expansion of a solid tumour, as may be used to induce tumour stasis or even assist in the prevention of micrometastases.

Further Differences Between Tumour Vascular Targeting and Destruction and Anti-Angiogenic Therapy

12. The concept of targeting the established, intratumoral vasculature within a solid tumour is fundamentally different to that of targeting the proliferating blood vessels at the periphery of the tumour mass. In recent years, it has become evident that the proliferating endothelial cells form only a small percentage of the tumour vascular endothelium, which argues against targeting against such proliferating cells as a means of causing significant anti-tumour effects. Results from studies conducted in my laboratory have shown that the proliferating endothelial cells within a solid tumour constitute only about 2% of the total endothelial cells within the tumour bed (Fox *et al.*, *Cancer Research*, 58:4161-4163, 1993; copy attached as **Exhibit E**). This figure is much higher than normal tissues (0.05%) and shows that tumour vasculature is proliferating at a higher rate than normal vasculature. However, it is only a small fraction of the vasculature in the tumour.

13. The studies published as Fox *et al.*, 1993 (**Exhibit E**) also revealed that tumour vascular endothelial cell proliferation occurred predominately at the tumour periphery, in contrast to the proliferation of the tumour cells themselves, which occurred throughout the lesion. Results from these studies led my colleagues and I to the conclusion that animal studies conducted prior to our publication in September, 1993, had significantly overestimated endothelial cell proliferation within tumours. There was no correlation between vascular endothelial cell proliferation and the extent of tumour vascularization, as measured by vascular density. This indicates that other mechanisms of remodelling of tumour vasculature are also important.

14. The importance of angiogenesis to tumour blood vessel growth has further been questioned in recent years, as our understanding of the importance of the process of intussusception has increased. Rather than growing only by the process of budding or sprouting capillaries, it is now accepted that tumour vasculature also develops by partitioning the vessel

lumen through the process of intussusception. Intussusceptive microvascular growth refers to vascular network formation by insertion of interstitial tissue columns, termed tissue pillars or posts, into the vascular lumen and the subsequent growth of these columns, resulting in a newly partitioned vessel (Patan *et al.*, *Microvasc. Res.*, 51:260-272, 1996; **Exhibit F**). It is now generally accepted that blood vessel growth within a tumour occurs by both endothelial sprouting and intussusception. Targeting only the budding capillaries will therefore not impact the majority of the vessels within a solid tumour. Furthermore, during the budding process, the initial sprouting vessels themselves have no lumen and are therefore not accessible to blood-bone agents, such as immunotoxins. This further hampers the ability to achieve significant anti-tumour effects using an anti-angiogenic strategy.

15. As anti-angiogenic strategies are intended to target proliferating vessels which are at the tumour periphery, such therapies may have potential for reducing the risk of micrometastasis, particularly upon repeat administration, and may also be used in inhibiting further growth of solid tumours. However, anti-angiogenic agents are unable to target the established tumour vessels that are composed of semi-dormant cells. In fact, evidence is that targeted therapy aimed only at the proliferating components will not result in tumour regression and anti-angiogenic agents are generally intended for use in small (< 1 mm) tumours.

16. As the established, intratumoral vessels are the lifeline of the tumour, only targeting and destroying vessels could reasonably be expected to result in tissue necrosis and concomitant tumour destruction. The anti-vascular strategies described in the referenced patent application provide for such tumour targeting and destruction. Representative studies have also been published by the inventors as the journal articles Burrows *et al.* (1992); Burrows and Thorpe (1993); and Huang *et al.* (1997) (**Exhibits B, C and D**). I distinctly recall reading these articles upon their publication, and being impressed by the data in these articles and being encouraged concerning the progress made by the inventors in bringing anti-vascular therapy forward as a realistic clinical treatment for use in patients with solid tumours. The *Cancer Research* article by

Dr. Thorpe and colleagues (**Exhibit B**) is considered by scientists working in this field as the first demonstration of "proof of principle" for targeting and destroying the established, intratumoral vasculature of solid tumours.

17. In summary, I conclude that the concepts of anti-angiogenic tumour therapy, as embodied in the Hagemeier and Thorpe references, are different from and do not suggest methods of targeting established tumour vasculature in order to induce necrosis and cause tumour regression, as in the invention. The strategies of anti-angiogenesis and vascular targeting are very different in that anti-angiogenesis is intended to prevent the growth, migration and formation of new blood vessels and that intratumoral vascular targeting is intended to destroy the established, blood transporting vessels. These strategies could therefore be effectively used in combination, with the anti-vascular therapy being used to reduce the tumour mass, followed by the use of anti-angiogenic therapy to prevent micrometastasis from any surviving tumour cells.

growth of

18. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the referenced application or any patent issued thereon.

16.6.97

Date

A L Harris

Adrian L. Harris, B.Sc., M.A., D.Phil., F.R.C.P.

H. Dec. Ex A

PROFESSOR ADRIAN L HARRIS

CURRICULUM VITAE 1997

NAME: Adrian Lewellyn HARRIS

ADDRESS:

N.H.S Address
ICRF Medical Oncology Unit
The Churchill Hospital
Headington
Oxford OX3 7LJ
Tel: (01865) 226184 FAX: (01865) 226179

Laboratory Address
ICRF Molecular Oncology Labs
Institute of Molecular Medicine
John Radcliffe Hospital
Headington
Oxford OX3 9DU
Tel: (01865) 222457 FAX: (01865) 222431

Home Address
83 Kingston Road
Oxford OX2 6RJ

DATE OF BIRTH: 10 August 1950

MARITAL STATUS: Married, 2 children. My wife is a General Practitioner.

CURRENT APPOINTMENT:

Imperial Cancer Research Fund
Professor of Clinical Oncology

Director of Molecular Oncology Laboratory
Institute of Molecular Medicine

Consultant Medical Oncologist
Oxford Radcliffe Trust

Fellow of St Hugh's College
University of Oxford

EDUCATION:

1960-1967	Liverpool Collegiate School
1967-1973	Liverpool University Medical School

QUALIFICATIONS:

1970	B.Sc. 1st Class Honours, Biochemistry, University of Liverpool
1973	M.B.Ch.B. Honours, University of Liverpool
1975	M.R.C.P. (UK)
1978	D.Phil., Corpus Christi College, University of Oxford. "The Clinical Pharmacology of Cytosine Arabinoside"
1985	Fellow of Royal College of Physicians
1989	M.A. Oxford University

ACADEMIC DISTINCTIONS:

1967-1970	Robert Gee Entrance Scholarship
1968	Distinction in Organic Chemistry
1969	Distinction in Biochemistry
1969	Augusta Lipkin Prize in Biochemistry
1970	Channon Prize in Honours Biochemistry
1971	J Hill Abram Prize in Pharmacology
	George Adams Prize in Pathology
	Kanthack Medal in Pathology
1972	Distinction in Pathology
1972	Distinction in Pharmacology
1973	Ernest F Reeve Prize in Mental Diseases
1973	Distinction in Medicine
	Distinction in Surgery
	Samuel Morris Green Prize in Surgery
	Owen T Williams Prize Clinical School Exhibition
	J Hill Abram Prize in Medicine
	Sir Robert Kelly Memorial Medal in Surgery
1986	Goulstonian Lecturer, Royal College of Physicians
1993	St Lukes Lecture and Medal
	Royal Academy of Medicine in Ireland
1994	Daniel E Bergsagel Visting Professorship, Ontario Cancer Institute, Toronto, Canada

PREVIOUS APPOINTMENTS:

Sept 1973 - Feb 1974	House Physician to Professor D A Price-Evans and Dr R B McConnell, Liverpool Royal Infirmary
Mar 1974 - Aug 1974	House Surgeon to Professor R Shields and Mr A Cushieri
Sept 1974 - Aug 1975	Senior House Officer to Dr T Bailey, Dr B Walker, Dr G Honey, Broadgreen Hospital (Diabetes, Cardiology, Geriatrics).
Sept 1975 - Aug 1978	Medical Registrar and Clinical Scientist (MRC Training Fellowship) in University Department of Clinical Pharmacology and Nuffield Department of Medicine, Oxford (Prof. D.G. Grahame-Smith, Prof D J Weatherall) (Clinical Pharmacology, Haematological Oncology, General Medicine)
Sept 1978 - Nov 1979	Medical Registrar in the Academic Department of Medicine, Royal Free Hospital London (Professor S Sherlock, Professor N McIntyre, Dr D Jewell, Dr I M James) (General Medicine, Gastroenterology, Clinical Pharmacology)
Nov 1979 - Nov 1981	Senior Medical Registrar, Royal Marsden Hospital, Fulham Road, London (Dr I E Smith, Dr E Wiltshaw). Lecturer, Institute of Cancer Research (Medical Oncology)
Nov 1981 - Nov 1982	Visiting research worker, Mutagenesis Laboratory, Imperial Cancer Research Fund, London (Dr T Lindahl)

Nov 1982 - Aug 1988 Professor of Clinical Oncology, University of Newcastle, Newcastle upon Tyne. Director, Cancer Research Unit, Royal Victoria Infirmary

ACCREDITATION: I have been accredited for Higher Medical Training in General Medicine and Medical Oncology since November 1981.

HONORARY NHS CONTRACT

6 sessions per week

DIRECTOR OF IMPERIAL CANCER RESEARCH FUND MEDICAL ONCOLOGY UNIT, OXFORD

The ICRF Medical Oncology Unit at Oxford comprises a purpose built clinical unit at the Churchill Hospital, and the Molecular Oncology Laboratories at the Institute of Molecular Medicine (IMM), John Radcliffe Hospital. The clinical unit formally opened in December 1992 by Her Majesty the Queen, includes a new ward (Blenheim) with 12 research beds and an out-patient department. There are three consultant Medical Oncologists (me, Dr D Talbot and Dr T S Ganesan).
I am also the Director of the ICRF Molecular Oncology Laboratories. There are four research teams in the Molecular Oncology Laboratory at the IMM; growth factors (AL Harris), cellular responses to DNA damage and drug resistance (I D Hickson, C Norbury), angiogenesis (R Bicknell), and ovarian cancer (T S Ganesan). Dr Talbot also has collaborations with the drug resistance group for preclinical pharmacology. Close links are maintained between the Molecular Oncology laboratory and the clinical departments. A summary of the research is appended -**Appendix 1.**

RESEARCH INTERESTS

My major interests are in tumour angiogenesis, mechanisms of its regulation and its role as a therapy target. I have a group of my own and interact on a regular basis with the other scienists running basic research in my unit to exploit clinically relevant areas. In the **Appendix I** therefore describe and list their projects , but highlight ones in which I am involved. Similarly to ensure day to day supervision of PhD students I jointly supervise with one of the scientists. In some cases they and in some I am the lead supervisor.

Breast cancer angiogenesis has been shown to be a major prognostic factor and growth factors regulating angiogenesis, mechanisms to inhibit them or exploit them therapeutically are a major area of work. Models and mechanisms of angiogenesis and ways of monitoring anti angiogenesis therapy in patients are being studied. The enzyme thymidine phosphorylase is upregulated 20 fold in breast cancer and is angiogenic by a novel pathway. We were the first group to show that the enzyme activity of thymidine phosphorylase is necessary for its angiogenic effects.

Topoisomerase IIβ was cloned by us and the roles of the different topoisomerases II are being studied in drug resistance and normal cell biology. The role of phosphorylation and relationship to drug resistance is being studied. We were also the first to clone the

topoisomerase II α promoter and have recently shown the regulatory functions during the cell cycle and confluence.

The human repair gene AP endonuclease has been shown to be relevant to repair from a wide range of DNA damaging drugs and also anti-metabolites. We were the first to show it has a major role in protecting cells from hypoxic cell death. Its role in regulating transcription factors and survival of hypoxic stress is being studied and attempts to make inhibitors ongoing.

Clinical trials involve several novel signal transduction modifiers as well as drugs to affect oxygen delivery to tumours and hypoxically activated agents. We were the first group to report on activity and biological assessment of the novel protein kinase C modulator Bryostatin in phase 1 and also on the left- shifting drug BW12c, which was monitored by magnetic resonance spectroscopy. Several drug resistance modifier studies are ongoing including continuous infusional therapy, novel drugs and liposomal drug delivery. A gene therapy programme has also started with a long term aim of vascular targeting, but initially developing experience in melanoma.

CLINICAL WORK

I direct and run the Medical Oncology service for NHS patients in the Oxfordshire region and for those adjacent regions with which we have contracts. Together we see 400 new referrals per year out of a total of 2,500 patients per year referred to the combined Radiation and Medical Oncolgy Department . We aim to provide a service integrating new discoveries from basic research with optimum conventional therapies. We work closely with the Radiotherapy Department with whom we collaborate on patient care, junior doctor and nurse training. We have cross cover and have joint contracts for providing cancer services. I do two clinics and two ward rounds per week and a 1 in 3 consultant rota for medical oncology patients. I personally see 200 new patients per year. I and two consultant colleagues, who also have laboratory commitments, provide the equivalent of 1.5 full time consultant sessions to the NHS. Tumour types for which medical oncology has a major role are the main workload, with breast cancer the commonest tumour type seen. Others include lung cancer, ovary, teratoma, lymphoma, colon, melanoma and renal cancer. Each consultant subspecialises and I run the breast cancer, melanoma and renal programmes.

PREVIOUS CLINICAL TRAINING

I trained in general medicine in Liverpool, Oxford and London, rotating through units with special interests in cardiology, diabetes, hepatology, gastroenterology, clinical pharmacology, geriatrics and clinical haematology. In all these jobs I was resident first on call for medical emergency admissions, on average 1 in 3. I was accredited as a specialist in General Medicine in 1981.
In my oncology training I did an MRC fellowship in Oxford where I looked after leukaemia and lymphoma patients for three years. In my hepatology job I treated hepatoma patients. At the Royal Marsden I was on the breast, lung, ovary and teratoma units. As a consultant in Newcastle I had large practices in breast, lung, colon,teratoma, lymphoma and ovarian cancer.

As a result of this range of experience , I have over the years published clinical papers on the following cancer types - breast, colon, glioma, hepatoma, leukaemia, melanoma, mesothelioma, myeloma, ovary, pancreas, sarcoma, small cell and non-small cell lung, teratoma .

BREAST CANCER GROUP

Breast cancer is treated by a multidisciplinary team comprising two breast surgeons (one woman surgeon), two radiologists from the breast screening programme, pathologist, cytopathologist, radiotherapist, medical oncologist (Prof A L Harris). The junior staff associated with the above specialities, nurses, counsellors, laboratory technical staff and other support staff also attend the weekly meeting where all the cases (benign and malignant) from the previous week are reviewed. Pathology, radiology and results from my laboratory on growth factor receptors and oestrogen receptors are available to optimise management. There are a range of routine and research protocols and written guidelines for management. Patients are seen for follow up at a joint breast clinic with a specialist surgeon, radiotherapist and medical oncologist (Prof A L Harris) present.

CLINICAL AND LABORATORY RESEARCH (see **Appendix1**)

I spend approximately half my time in directing laboratory research and half on clinical work and research. My aim is to carry out basic laboratory research in areas of potential clinical importance and to try and apply this in the clinic to develop new treatment approaches. This applies particularly to breast cancer but also other tumour types described above.
I produce a report each year for the ICRF, summarising progress in these areas in the laboratory I direct at the Institute of Molecular Medicine. (see **Appendix 1**).
Areas of particular relevance to breast cancer include:-

- breast tumour angiogenesis
- prediction of prognosis
- new targets for therapy, particularly signalling kinases
- drug resistance mechanisms
- therapy studies in early and recurrent breast cancer
- neoadjuvant treatment

GRANTS AND FUNDING

The Imperial Cancer Research Fund provides £1,760,000 per year to run the research. This is subject to quinquennial review. The NHS provides £900,000 per year which covers service costs. I obtain funding on a regular basis from the pharmaceutical industry - on average £200,000 per year. I regularly obtain grants for clinical training fellowships from the MRC- average grant £35,000 year. In the last 2 years the following projects were funded:

- Antisense IGF I for gene therapy (4 years).
- Isolation of peripheral blood dendritic cells and pulsing with acid eluted peptides (3 years).
- Hypoxia activated constucts for gene therapy (3 years).

ADMINISTRATION

Royal College of Physicians
Medical Oncology Advisor (1990 - 1993)
Medical Oncology site visitor (1996-)

NHS Administration
Acute Sector Panel Standing Group on Health Technology (London)
Arts Committee Churchill Hospital
Cancer Centre Clinical Board
Cancer Centre Steering Committee
Service Delivery Unit Chairman - Medical Oncology

University Administration
Board of the Faculty of Clinical Medicine
Lee Placito Cancer Research Fund
Review Committee Research Institute

MEMBERSHIP OF PROFESSIONAL SOCIETIES

American Association for Cancer Research
American Society of Clinical Oncology
Association of Cancer Physicians
British Association for Cancer Research
British Breast Group
British Oncology Association
EORTC Pharmacokinetics and Metabolism Group
Royal College of Physicians

ICRF COMMITTEES AND GROUPS

There are a number of ICRF Committees necessary for integration of laboratory and clinical work and optimising collaborations. I am a member of the following groups or committees.
ICRF Breast Cancer Group
ICRF Clinical Research Committee
ICRF Clinical/Laboratory Strategy Committee
ICRF Developmental Therapy Group
ICRF Gene Therapy Group
ICRF Growth Factor Group
ICRF Melanoma Group

SCIENTIFIC ADMINISTRATION

Cancer Research Grant Award Committees and Advisory Boards

Antisoma Biotechnology Advisory Committee
Bath Cancer Research Unit Scientific Committee
Beatson Cancer Research Institute Advisory Committee
British Technology Group: New Cancer Product Development Advisory Board

Cancer Research Campaign Phase I/II Committee (1981-present)
Cancer Treatment Research Foundation - Board of Scientific Counsellors
Clatterbridge Cancer Research Trust
EORTC Urological Group (1981-1988)
KS Biomedix Scientific Advisory Board
Leukaemia Research Fund (1987-1989)
Medical Research Council Cell Board A Grants Committee (1985-1989)
MRC Cancer Therapy Working Party (1985-1989)
Royal Marsden Hospital Scientific Advisory Board
Xenova Board of Directors
Scientific Board of Institut Curie - oncology conference 1997 -

Grant Assessor for following Boards

Association for International Cancer Research
Cancer Council - Australia
CRC Project Grants
Dutch Cancer Society
Leukaemia Research Fund
MRC Training Fellowships
MRC Grants
NIH Grants Review Branch
North of England CRC
Hong Kong University

Site Visits

1991
CRC, Glasgow CRC, Charing Cross
1993
CRC, Gray Laboratory
1996
CRC, Birmingham

Promotion Assessments

Georgetown University, Washington
National Cancer Institute, Washington
Princess Margeret Hospital, Toronto
University of London

Examiner: MD and PhD Degrees

Since 1992 I have examined 6 - MD/DM theses and 10 - PhD/DPhil from the following Universities:- London, Oxford, Dundee, Nottingham, Liverpool, Cambridge .

Editorial Board of following journals
Anti-Cancer Drugs
British Journal of Cancer
Cancer Surveys (Associate Editor)
Cancer Topics
Cancer Topics Abstracts Service
Clinical Cancer Research
Current Medical Literature : Breast and Prostate Cancer
European Journal of Cancer (until July 1995)
Gene Therapy of Cancer

Oncology Today
Oxford Textbook of Oncology
Referee for following journals

Acta Oncologica
American Journal of Gastroenterology
Biochemical Pharmacology
Breast Cancer Research and Treatment
Cancer Chemotherapy and Pharmacology
Cancer Communications
Cancer Letters
Cancer Research
Carcinogenesis
Clinical Chemistry
Clinical & Experimental Metastasis
Eur J of Cancer and Clinical Oncology
FEBS
International Journal of Cancer
Journal of Endocrinology
Lancet
Nature
Nature Genetics
Nature Medicine
The Breast
Theoretical Surgery

EXTERNAL REVIEWS OF THE UNIT

In 1994 my unit was reviewed by three different teams. These reviews are available.

(1) **University Review (Review of clinical, teaching and research activities of the University Department of Clinical Oncology and Future Directions)**

This comprised members of other departments and other universities. The specific comments were as follows:-
"The Review Committee was extremely impressed with the way in which the unit and laboratories had been set up and were functioning, bearing in mind the start date of the activities".

(2) **Royal College of Physicians Review (Assessment of Postgraduate Training, Accreditation of Unit, Clinical Practice)**

This review stated:-
"The environment for this post is a model for the type of broad exposure needed for training medical oncology, with concentration on cancer not burdened by peripheral duties, poor unit structure or administrative work".

(3) **Imperial Cancer Research Fund Quinquennial Review (Assessment of laboratory and clinical research)**

A committee of 12 scientific and 2 clinical assessors reviewed the last five years clinical/laboratory research and the next five years plans. The majority of the reviewers were from outside the United Kingdom. Extremely high ranking by international standards was achieved and the next five years funding was recommended.

REFEREES:

Dr I E Smith
Consultant Medical Oncologist
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London
SW3 6JJ

For my time since 1989 at the ICRF

Professor T Lindahl
Imperial Cancer Research Fund Laboratories
Clare Hall Laboratories
Blanche Lane
South Mimms
Potters Bar
Herts EN 6 3ZD

Professor S.B. Kaye (external reviewer on 1994 site visit)
CRC Dept. of Medical Oncology
Alexander Stone Building
Garscube Estate
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Bearsden
Glasgow G61 1BD

Professor N.Wright
Director of Clinical Research
Imperial Cancer Research Fund
44 Lincoln's Inn Fields
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APPENDIX 1
CLINICAL AND LABORATORY RESEARCH PROGRAMME

Medical Oncology Unit (Churchill Hospital, Oxford)

Overview and project reports for clinical unit

Clinical studies include assessment of drug resistance mechanisms in common cancers such as breast, lung, colon, melanoma and hypernephroma. These include topoisomerase II and apoptosis pathways. Under trial are agents that either modify DNA repair or inhibit multidrug resistance and evaluation of schedule-directed drug administration. Other studies involve the*in vitro* evaluation of new drugs with novel mechanisms of action prior to entry into clinical studies.

Gene therapy is a novel therapeutic approach to treating common solid tumours. Projects based on promoters that are activated in specific tumour types have begun. Initial work involves melanoma and the IL-2 gene (in collaboration with Dr. Ian Hart).

Growth factor receptor levels are measured in breast cancer biopsies and the data is used to select high risk patients for therapy. Growth factor receptors and their signalling pathways are also used as potential targets for therapy with novel agents.

Angiogenesis is a critical step in tumour development, but often the blood supply to tumours is inadequate. Some drugs are more active under hypoxic conditions, and thus we are using new agents which modify tumour oxygenation (monitored by magnetic resonance spectroscopy or other markers of angiogenesis) and drugs activated by hypoxia. Novel drugs that inhibit angiogenesis are being assessed in early studies.

Apart from work geared to improving the effectiveness of antitumour therapy, we are undertaking a number of studies which address quality of life problems, including the use of new antiemetics, studies on the mechanism of fatigue induced by chemotherapy.

1. **A phase IB dose escalation study of CGP 41251 in patients with advanced cancer**
   D Propper, K J O'Bryne, D C Talbot, A L Harris

CGP 41251 is a protein kinase C antagonist and rverses multidrug resistance. It has shown significant antitumour activity, both in vitro against malignant cell lines, and *in vivo* against a range of human tumours xenografts. We have commenced a Phase I dose escalation study in patients with solid tumours.

2. **Pre-clinical pharmacology - enzymes for gene therapy**
   A Patterson, S Houlbrook, D C Talbot, A L Harris,
   I Stratford *MRC Radiobiology Unit, Harwell*

The cytotoxic action of new clinical drugs is being assessed on a range of lung and breast cancer cell lines, including a number of multidrug resistant cell lines. This provides essential data for design of clinical trials. Hypoxically activated cytotoxic drugs may

synergise with antiangiogenic therapy. SR4233 is one of this class of agents under study. We have shown that sensitivity of human breast cancer cell lines to this drug is directly related to the activity of P450 reductase. High expression of these genes in tumours could lead to selective drug activation and synergy with radiation or chemotherapy. Thus, these genes driven by tumour specific promoters, may be suitable candidates for gene therapy. Thymidine phosphorylase , which activates the prodrug capcetabine, is being studied in transfected cell lines and xenografts.

3. **Continuous infusional etoposide phosphate in the treatment of small cell lung cancer**
D C Talbot, K O'Bryne, H Robertshaw

Etoposide is a highly schedule dependent drug and is the most commonly used drug in the treatment of small cell lung cancer. As etoposide has poor aqueous stability and solubility, we are evaluating the etoposide pro-drug, etoposide phosphate given by continuous ambulatory intravenous infusion. This is a randomised study in collaboration with St Bartholomew's Hospital and is comparing the efficacy of etoposide phosphate given by pharmacokinetically guided constant infusional dosing, targeting a plasma etoposide concentration of 1mg/ml for 15 days or 3 mg/ml for 5 days.

4. **Assessment of *in vivo* DNA damage following treatment with drugs**
S Houlbrook, J Braybrooke, A L Harris, D C Talbot

Many cytotoxic drugs exert their clinical effect by inducing DNA strand breaks. The aim of this study is to determine the degree of strand breaks generated in tumours and peripheral lymphocytes *in vivo* in patients treated with DNA-damaging drugs including dacarbazine and procarbazine. We have compared the rate of strand break formation to the degree of AP endonuclease activity expressed by these tissues. Two methods have been used to assess DNA damage 1) The "comet" assay in which alkaline-labile DNA strand breaks are quantified in individual cells by examining by confocal microscopy the extent of DNA migration from the nucleus, and 2) the amount of DNA damage repairable by HAP 1 as measured by incorporation of ^{32}P-dCTP into DNA. The ability to measure such pharmacokinetic endpoints *in vivo* will allow investigation into the effects of new therapies and help in the design of drug schedules in combination chemotherapy.

5. **Phase III trials with taxol, capcetabine and losoxantrone**
D Propper, J Braybrooke, K O'Byrne ,T S Ganesan, D Talbot, A L Harris

In randomised trials in breast cancer we are assessing activity of taxol versus capcetabine after adriamycin resistance, and losoxantrone as a less toxic analogue.
versus adriamycin

6. **Gene therapy with tissue specific promoters**
D Propper, A L Harris, D Talbot, R Bicknell
F Balkwill *ICRF Labs, Lincoln's Inn Fields, WC2A 3PX,*
I Hart *ICRF Labs, Lincoln's Inn Fields, WC2A 3PX,*
T Seddon *ICRF Clare Hall, Potters Bar EN6 3LD*

Selective expression of IL-2 within melanomas may be achieved by using the tyrosinase promoter coupled to the IL-2 gene. Phase I studies have started with direct injection of constructs into skin secondaries, to evaluate regulation and expression of the gene construct in human disease.

7. **Gene therapy - dendritic cell isolation**
D Chao *Nuffield Dept of Surgery John Radcliffe Hosp,*
J Austyn *Nuffield Dept of Surgery, John Radcliffe Hosp,*
A L Harris

For successful gene therapy, it is likely that tumour antigen presentation via class I MHC molecules is essential. Dendritic cells are being isolated from peripheral blood stem cells and methods of eluting tumour antigens from biosies evaluated for use in immunisation trials.

8. **Gene therapy - low dose interferon-g and *in situ* cytokine expression**
TS Ganesan, A L Harris
L Kaklamanis *Nuffield Dept of Pathology, John Radcliffe Hosp,*
F Balkwill *ICRF Labs, Lincoln's Inn Fields WC2A 3PX*

Suppression of class I MHC and TAP1 in tumour cell lines can be reversed by interferon-g*in vitro*. Assessment of this process *in vivo* is the subject of a phase II trial in melanoma to see if suppression can be reversed. If so, this approach may be synergistic with gene therapy using IL-2.

9. **EGF Receptor antibodies**
K O'Byrne, D Talbot, A L Harris, S Dorrington K. Smith

I^{123} labelled EGFR1, a monoclonal antibody to the EGF receptor external domain is being evaluated in patients with resectable non-small cell lung cancer. If a significant localisation of antibody can be achieved, this may be useful for targeting to tumours that express high levels of EGF receptor.

10. **Cyclic AMP analogues to inhibit tumour growth**
D Propper, A L Harris, D Talbot, T S Ganesan
Y S Cho-Chung *NIH, Bethesda, Maryland 20892, USA,*

Cyclic AMP dependent protein kinase regulatory subunits are often over-expressed in human cancers. Drugs that differentially inhibit type I or activate type II cause differentiation and inhibit growth. A Phase I trial is in progress using a continuous infusion of 8 Chloro cyclic AMP.

11. **Angiogenesis as a Prognostic Marker**
S Fox *Nuffield Dept. of Pathology, Univ. of Oxford,*
K Gatter *Nuffield Dept. of Pathology, Univ. of Oxford,*
M Greenall *Nuffield Dept. of Surgery, Univ. of Oxford,*
R Leek, A L Harris

In order to assess the vascularisation of tumours, a range of antibodies have been examined on paraffin sections. CD31 was the best marker for capillary vessels. We have been able to quantify the degree of vascularisation of tumours, and are relating this to the expression of angiogenic factors within the tumours. Initial studies with breast tumours show that the degree of capillary vascularisation is associated with the grade of tumour, larger tumour size, and lymph node metastasis. Similar results have been affirmed with NSCCC. Its value particularly in node negative patients and as a marker of hormone resistance is being studied. It appears to be a major prognostic factor and we are planning to use it for selecting patients for more intensive therapy. Similar results have been found in lung and bladder cancer.

12. Metalloproteinase inhibitor BB94: Phase II study in patients with malignant pleural effusions

D C Talbot, K O'Bryne, T S Ganesan, A L Harris

BB94 (Batimastat) is a matrix metalloproteinase inhibitor which has been developed to disrupt growth and regional spread of solid cancers and to block the process of tumour neo-vascularisation. Pre-clinical studies have confirmed its anti-tumour effectiveness, primarily in the ovarian xenograft model. We have completed a Phase I study evaluating the pharmacokinetics safety and tolerability of intrapleural Baltimastat in patients with malignant pleural effusions. Batimastat had a long half life following intrapleural administration resulting in plasma levels exceeding those required for *in vitro* anti-tumour effects for protracted periods after a single administration. The current study is evaluating efficacy e.g. intrapleural Batimastat in management of patients with malignant pleural effusions.

13. A Phase II study of ICRF 159 in renal cell carcinoma

K J O'Bryne, D Propper, J Braybrooke, T S Ganesan, D C Talbot, A L Harris

ICRF 159 is a topoisomerase II inhibitor with anti-angiogenic properties. Renal cell carcinomas are highly angiogenic tumours. The purpose of the present study is to evaluate low dose ICRF 159 in the treatment of renal cell carcinoma. We are monitoring changes in blood flow in the tumours using colour flow doppler. We are also evaluating the role of potential plasma and serum angiogenesis markers, including von Willebrand's factor levels, in monitoring response therapy.

14. A Phase I study of interferon a for induction of thymidine phosphorylase, in the treatment of solid tumours

M Koukourakis *Visiting Honorary Senior Registrar, Churchill Hosp*
K O'Bryne, D Propper, J Braybrooke, T S Ganesan, D C Talbot, A L Harris

We have commenced a Phase I dose escalation study using interferon 2a, in combination with standard two day 5-FU and folinic acid infusion therapy. Interferon may enhance 5-FU through induction of thymidine phosphorylase which converts 5-FU to active metabolites. Based on the *in vitro* work the optimal time for enhancing thymidine phosphorylase activity may be prior to administration of the 5-FU. Therefore, we are administering inferon a in increasing doses and for increasing

duration of time prior to 5-FU and folinic acid therapy and monitoring in vivo induction of the enzyme.

15. **A Phase I/II study of hydroxyurea in combination with 5-fluorouracil and folinic acid in the treatment of colorectal carcinoma**
K O'Bryne, D Propper, J Braybrooke, T S Ganesan, D C Talbot, A L Harris

Hydroxyuea is an inhibitor of ribonucleotide reductase and as such may reduce the pool of nucleotides available for repair of the DNA damage induced by 5-FU and folinic acid. We are evaluating the efficacy of this combination in a Phase I/II study in colorectal cancer.

16. **Thalidomide and Carbolatin in ovarian cancer**
M Saunders, K O'Byrne, A L Harris,T S Ganesan

Thalidomide has been recently shown to have anti-angiogenic activity in preclinical studies. A phase II study to evaluate the safety of administering this together with Carboplatin and evaluating the synergy has been initiated. This is potentially a new approach to treating cancer by compounds which inhibit tumour vascular supply in combination with chemotherapy.

Molecular Oncology Laboratory: Genome Integrity Group

(i) Chromosome Transmission and Stability - Dr. Ian D. Hickson

Overview and project reports

This group has two main interests; (i) the roles of DNA topoisomerase II in DNA metabolism and as a target for antitumour drugs (ii) analysis of human genes required for DNA base excision repair.

Several aspects of topoisomerase II biology are being investigated, using either yeast or mammalian cell lines as model systems. In yeast, we are using the two-hybrid cloning system to identify new components of the nuclear machinery which are required for chromosome segregation during cell division. In contrast to yeast, mammalian cells express two closely-related isozymes of topoisomerase II, designated a and b. In human cells, we are analysing the role of phosphorylation in regulating topoisomerase II isozyme activity at mitosis and the mechanisms which underly the cell cycle dependence of topoisomerase II gene expression. Topoisomerase II is one of the major cellular targets for some of the most widely used and effective antitumour agents. We have a number of ongoing studies aimed at identifying the importance of the a and b isozymes as targets for different classes of drugs, and ways in which we might improve therapy with topoisomerase II-targeting drugs.

Our DNA repair studies focus primarily on the nuclear enzyme HAP1, which is the major endonuclease in human cells which repairs DNA containing baseless sites, or other lesions generated by reactive oxygen species. These species are generated by x-rays

and many classes of DNA damaging drugs. Failure to repair these lesions is both mutagenic and toxic, and may be a factor in cellular transformation and ageing.

1. **Post translational modification of human topoisomerase II a and b proteins**

2. **Role of topoisomerase II in chromosome segregation and genome stability**

3. **Use of the two hybrid cloning strategy to identify proteins which interact with the C-terminal domain of eukaryotic topoisomerase II**

4. **Analysis of mammalian cell mutants hypersensitive to topoisomerase inhibitory drugs**
   SL Davies, PS North, A L Harris, I D Hickson,

A range of studies on CHO cell mutants is ongoing to identify the genes controlling cellular sensitivity to topoisomerase inhibitors. Two mutants show no apparent abnormality in topoisomerase II enzyme expression but show an altered response to agents that modulate cAMP dependent protein kinase. One of these mutants overexpresses the RIa subunit of protein kinase A. A cDNA library has been transfected into this mutant and the phenotype reverted. The integrated cDNA has been rescued from the transfectant and is currently being analysed. The mutant ADR-3 is cross resistant to the topoisomerase I inhibitor, camptothecin and shows a defect in the repair of x-ray induced DNA strand breaks. ADR-3 also appears to exhibit an abnormality in chromosome segregation and displays aneuploidy, possibly indicating a defect in topoisomerase II. Chromosome segregation analyses are being conducted on this mutant.

5. **Regulation of topoisomerase II gene expression in human cells**
   R Isaacs, M I Sandri, A L Harris, I D Hickson

Topoisomerase IIa is a cell cycle regulated protein in mammalian cells and expression falls rapidly at the onset of quiescence. The level of topoisomerase II protein may be of clinical relevance since this is one of the key determinants of cellular sensitivity to many important antitumour agents including adriamycin, VP16 and mitoxantrone. The expression of the gene peaks in the G2/M phase of the cell cycle. Dissection of the sequences important for the cell cycle regulation of gene expression is in progress using promoter fusions to the growth hormone gene. The necessary elements have been localised to a 200 bp region of the promoter. A similar approach is being used to study regulation of topoisomerase IIa gene expression by oncogenes and the mechanism of down regulation at confluence. Cell lines showing resistance to topoisomerase II inhibitors have been shown to express reduced topoisomerase II mRNA. The mechanism for this is also being investigated.

8. **Analysis of a human DNA repair enzyme**

9. **Role of the HAP1 protein as a redox regulator of transcription factors**

10. **Role of the HAP1 protein in protection against DNA damaging agents and hypoxic stress**

(ii) Cell Cycle Checkpoints and Apoptosis - Dr. Chris Norbury

Overview and project reports

DNA damage is fundamental to many forms of cancer therapy, and can induce two contrasting cellular responses. "Checkpoint" controls arrest the cell cycle in the G1, S or G2 phases after DNA damage, potentially allowing DNA repair and cell survival. In multicellular organisms DNA damage can also induce apoptotic cell death; successful cancer therapy requires that the apoptotic response predominates over cell cycle arrest and repair. We aim to understand the molecular mechanisms that underlie checkpoint controls in human cells, and that determine the balance between cell death and survival following DNA damage.

1. **Cell cycle regulators and the onset of apoptosis**
   W Ongkeko, A L Harris, C Norbury

We have investigated the proposition that the processes of mitosis and apoptosis might share common biochemical mechanisms. Contrary to the findings of other groups, we find that the mitosis-promoting protein kinase Cdc2 is not required for apoptosis, suggesting that the chromatin condensation that accompanies both processes is brought about by distinct routes. Surprisingly, inhibition of Cdc2 activity dramatically enhanced the level of apoptosis resulting from exposure to DNA damaging anticancer drugs. Our results suggest that a form of Cdc2 present in interphase cells is capable of suppressing the onset of apoptotic death in response to DNA damage. This form of Cdc2 would delay death in cells arrested at the G2 cell cycle checkpoint, thus favouring DNA repair and cell survival. We are currently investigating the biochemical details of this phenomenon, and characterising the synergistic effects of chemical inhibitors of Cdc2 and DNA damaging drugs.

2. **Identification of novel mammalian checkpoint components**

3. **A human homologue of fission yeast *pad1***

Molecular Oncology Laboratory: Growth Factor Group - Adrian L. Harris

Overview and project reports

1. **Breast cancer, angiogenesis and growth factors**

The aim of this group is to carry out basic studies relating the biology of breast cancer to development of new therapies and understanding the variability in clinical behaviour of the disease. Angiogenesis is a critical step in tumour growth and metastasis and the factors that induce it early on in the progression of breast cancer from *in situ* to invasive tumours are being studied. This may be relevant to breast cancer prevention in the treatment of patients with high risk of breast cancer and in patients with ductal carcinoma *in situ* to prevent invasive tumours developing.

Because of the importance in angiogenesis in poor prognosis via early metastasis and resistance to hormone therapy, mechanisms of angiogenesis are being studied to develop new therapy approaches. There are marked differences in the inducibility of angiogenic factors in different cell lines by hypoxia. The pathways concerned are being investigated. Paracrine interactions are known to be important in angiogenesis in normal tissue development and we have recently the role in macrophages in human breast cancer angiogenesis.

Understanding the normal biology and development of the breast may contribute to the understanding mechanisms involved in tumour progression and variable biological behaviour of breast cancer. Wnt genes and notch analogues are developmental genes in Drosophila that have been shown to act as oncogenes in mouse breast cancer and have major roles in embryonic development. We are evaluating their roles in human breast cancer.

2. **Role of wnt genes in human breast cancer: Wnt regulation, mechanisms of action and functions**
T D Bui, K Smith, S Dorrington, R Bicknell, A L Harris
M Jonsson, *Dept Oncology, Lund University Hospital, Sweden*

In order to evaluate further their roles in breast disease, we have investigated the expression of several members of the wnt gene family in primary breast cancer, normal and benign tissues and in breast cancer cell lines. There were major differences in genes expressed and wnt7b was upregulated in a subset of breast tumours. Wnt5a was up to 20-fold overexpressed in tumours. In contrast, wnt4 and 2 were upregulated in fibroadenomas.

3. **Isolation of novel wnts**

In order to investigate other novel wnts that may be upregulated in breast cancer, PCR using conserved primers was used and 3 further human wnt genes were isolated. One is previously undescribed in any species, the others are human homologues of mouse genes. There is evidence that one of these is overexpressed in a small subset of breast tumours. We have proceeded to investigate factors that regulate wnt expression, wnt signalling pathways and mechanisms of their effect. Based on embryonic studies, they may have a role in tissue remodelling and cell migration.

4. **Regulation of wnts**

The human breast cell line HB2 shows 10 fold upregulation of wnt 5a at confluence or during increases in cell density .The cell adhesion pathways involved are being studied.

5. **Function of wnts**

To investigate signalling pathways, inducible constructs of the wnt genes most frequently upregulated in breast cancer are being produced and are being assessed in the HB2 breast cell line. The pathways to be investigated, include dishevelled and cell adhesion signalling pathways. Conditioned medium from Drosophila cells

overexpressing Drosophila wnt1 or human wnts is being used as a source of soluble wnt protein to investigate transient signalling pathways.

6. **Macrophages and breast cancer angiogenesis**

R Leek *Nuffield Dept of Pathology, John Radcliffe Hosp*
C Lewis *Nuffield Dept of Pathology, John Radcliffe Hosp*
A L Harris

Macrophages produce many angiogenic factors and these may be induced under hypoxia e.g. basic FGF and VEGF. We have developed the methods for quantitating macrophage numbers in human primary breast cancer and shown there is a significant correlation with high angiogenesis. The macrophages are associated with necrotic areas and also can produce VEGF. The mechanisms by which macrophages survive severe hypoxia and induce VEGF are being studied .

7. **Breast cancer angiogenesis and *in situ* carcinoma**

S B Fox, K C Gatter, R Bicknell, A L Harris
K Engels *Dept Obstetrics & Gynaecology, Dusseldorf Univ*
N Bundred *University Hospital of South Manchester*

In situ breast cancer of different phenotypes has different risks of developing into invasive breast cancer.. The tumour vasculature is being studied and shown to have different phenotypes in high and low risk *in situ* cancer. The flt4 receptor is a receptor for a novel member of the VEGF family and is beng assessed . Clinical trials are ongoing using inhibitors of collagenases and demonstration of the role of proteases in progression of *in situ* breast cancer could suggest their potential as a target for prevention therapy.

Growth of *in situ* breast cancer in nude mice provides a model for investigating the roles of angiogenic factors in tumour progression. Using expression vectors being developed for gene therapy, we are transfecting the individual angiogenic factors we have previously shown to be associated with poor prognosis and to be markedly upregulated in human breast cancer.

8. **Apoptotic pathways and drug resistance in breast cancer**

Janet Neale *Visiting Worker, Oxford Health Authority*
K Gatter *Nuffield Dept of Pathology, John Radcliffe Hosp*
R Isaacs, K Vessey, A L Harris

Although growth factors and receptors in the tyrosine kinase pathway are important in breast cancer behaviour and responsiveness to therapy, their interaction with genes producing apoptosis is likely to be critical in the final outcome. We have previously shown that BCL2 is present in normal breast tissue and breast cancers but is lost in the more aggressive phenotypes. Surprisingly, loss of BCL2 was associated with resistance to adjuvant CMF and adjuvant tamoxifen when one would have expected that the presence of BCL2 would cause resistance and protect against apoptosis. We are therefore investigating the potential role of BCL2 expression in radiation resistance and local recurrence of breast cancer and the interactions with topoisomerase II inhibitors in

producing remission in advanced breast cancer. In the same cases the topoisomerase IIa expression is also being studied.

Molecular Oncology Laboratory: Angiogenesis Group - Dr. Roy Bicknell

Overview and project reports

Angiogenesis (the growth of new blood vessels) is a rare event in healthy adults, occurring only during wound healing and in the ovary and endometrium during the menstrual cycle. Over the past twenty years, substantial evidence supporting the hypothesis that the growth of solid tumours is dependent upon the ability of the tumour to elicit growth of a blood supply has accumulated. High vascular density of tumours has been shown by several groups to be an independent prognostic factor in a number of solid tumour types, including breast, bladder, prostate and head and neck cancer.

Angiogenesis is a feature of all solid tumours and as such offers a widely applicable opportunity for therapeutic intervention. Approaches to controlling angiogenesis have to date attempted to inhibit growth factor binding to receptors, growth factor signalling pathways or proteases involved in vascular invasion. Our studies are aimed at elucidation of the mechanisms of tumour angiogenesis and of novel ways in which this may be controlled.

The other interest of our group is vascular targeting as an anti-cancer strategy. This attempts to exploit differences between tumour and normal vasculature in order to target toxins to a tumour. It has been shown by others to be an effective anti-tumour strategy in animal models. Vascular targeting should be distinguished from the inhibition of angiogenesis, since the former aims to directly destroy tumour vasculature via a variety of cytotoxic agents, whereas the latter involves inhibition of endothelial migration and growth.

1. **Transfection of angiogenic factors into MCF-7 breast carcinoma cells. Effects on tumour growth**

2. **$VEGF^{121}$ transgenic mice**
   HY Chan, AL Harris, R Bicknell

To examine the effect of overexpression of an angiogenic factor in epithelial tissues on development and potentially tumorigenesis, we have developed a transgenic model with $VEGF^{121}$ expression under control of either the mucin-1 (muc-1) gene promoter for widespread tissue expression or the MMTV promoter for local high level expression in the breast.

3. **Elucidation of the mechanism of the angiogenesis induced by platelet-derived endothelial cell growth factor/thymidine phosphorylase**

4. **Midkine and pleiotrophin are angiogenic and promote tumour growth *in vivo***

5. **Role and regulation of the different isoforms of vascular endothelial growth factor.**
PAE Scott, H Turley, AL Harris, R Bicknell
KC Gatter *Dept of Cellular Science, University of Oxford*

Vascular endothelial cell growth factor (VEGF) is a potent inducer of angiogenesis *in vitro* and *in vivo*. There are four isoforms, each with varying effects on endothelial cell growth and vascular permeability. Three isoforms have been detected in a large number of malignant cell lines and tumours. We have shown that high expression of VEGF 121 in breast cancer is associated with poor prognosis. Xenografts with high VEGF 121 grow more rapidly than those with other isoforms and the role of proteases interacting with these isoforms is under study. In human breast cancer cell lines there is a 10 fold variation in VEGF induction under hypoxia and hypoxia signalling pathways that may cause this are being studied.

6. **The mechanism of endometrial angiogenesis**

7. **Tamoxifen stimulated growth of normal human endometrial isolates and the isolation of oestrogen and tamoxifen induced genes in endometrial tissue by PCR display**

8. **Vascular targeting of retrovirus-mediated gene therapy**
R T Jaggar, H Y Chan, S Morgan, A L Harris, R Bicknell
T Friedman *Visiting Sabbatical Professor, Univ of Calif, La Jolla*

We are attempting to target a marker gene (lacZ), a gene toxic to tumour vasculature (hTNF-a) and a pro-drug activating gene (thymidine phosphorylase/TP) to the tumour vasculature via retrovirus-mediated gene transfer. We have produced expression vectors for these genes in both plasmid and retroviral form using a constitutive promoter (retroviral LTR of pBABEpuro) and the E-selectin promoter, which is activated in the endothelium of both breast tumours and melanomas. We have packaged the retroviral vectors in both ecotropic and amphotropic forms and viral titres are of the same order as has previously been published. We have successfully infected large vessel- and small vessel endothelium *in vitro* with these constructs and are currently analysing expression patterns in these cells. We have also set up animal models to assess the effects of the various amphotrophic and ecotrophic constructs on growth of xenografts .

9. **Growth factor targeting of pseudotyped retrovirus to tumour endothelium**
H Y Chan, A L Harris, R Bicknell
H A Weich *G.f. Biotechnologische Forschung, Braunschweig, Germany*
T Friedmann *Visiting Sabbatical Professor, Univ of Calif, La Jolla*

The receptors for the endothelial-specific growth factor VEGF (KDR and flt-1) are known to be induced by hypoxia and highly expressed on tumour endothelium. We are evaluating the effectiveness of using biotinylated recombinant VEGF, together with avidin, biotinylated anti-VSVG antibody and pseudotyped retrovirus (with b-gal reporter) to target virus delivery to endothelium *in vitro* and *in vivo*.

10. Cloning of the KDR promoter for use in vascular targeted gene therapy
H Y Chan, AL Harris, R Bicknell

KDR is one of the receptors for vascular endothelial growth factor. The expression of KDR is restricted to endothelium and beta islet cells in the pancreas. KDR expression is induced by hypoxia. Hence the KDR promoter could provide specificity for vascular targeted gene therapy. We have cloned the promoter and used it to create several retroviral vectors regulating TP and TNF a.

11. Gene therapy using hypoxia promoters.

A Patterson	*MRC Radiobiology Unit, Harwell, Oxon*
M Saunders	*MRC Radiobiology Unit, Harwell, Oxon*
G Dax	*MRC Radiobiology Unit, Harwell, Oxon*
I Stratford	*MRC Radiobiology Unit, Harwell, Oxon*
P Ratcliffe	*Haematology Lab, Institute of Molecular Medicine*
J Firth	*Haematology Lab, Institute of Molecular Medicine*
C Pugh	*Haematology Lab, Institute of Molecular Medicine*
R Bicknell, A L Harris	

Hypoxia enhancer elements from the phosphoglycerate kinase gene have been ligated to a series of promoters which as a result are upregulated in function under hypoxia. This approach has been used to switch on pro-drug activation enzymes in hypoxic areas of tumours. Since the pro-drugs already show a selective toxicity of 20-30 fold between hypoxic tumours and normally oxygenated areas, the further upregulation of the enzymes that activate these drugs may produce up to 200-fold differential sensitivity and cytotoxicity. Using diffusible pro-drugs the effect can extend to better oxygenated areas of tumour and may greatly enhance selective toxicity and drugs against cancer. *in vitro* and *in vivo* experiments have verified that this approach is feasible and current projects involve investigating a range of pro-drug activation systems, including thymidine phosphorylase, activation of cyclophosphamide and interactions between the hypoxia response elements and other tissue specific promoters or enhancers. Novel hypoxia enhanced drugs are being evaluated as are the enzyme systems that activate them.

12. Thymidine phosphorylase as a mechanism for gene therapy and ADEPT
A Patterson, I Stratford, R Bicknell, D Talbot, A L Harris
W Gullick *ICRF Molecular Oncology Labs, Hammersmith Hosp*

It has been shown that thymidine phosphorylase activates the pro-drug furtulon and this can kill cells by a bystander effect that does not require gap junctions. This has a great advantage over other types of antimetabolite activation. Because thymidine phosphorylase requires no co-factors its role as an extracellular enzyme activating furtulon is being assessed and recombinant forms are being produced for targeting to growth factor receptors by combining single chain variable domains with the enzyme.

13. Bladder cancer angiogenesis - mechanisms of regulation and role in tumour progression

J Crew	*Department of Urology, Churchill Hospital*
D Cranston	*Department of Urology, Churchill Hospital*

S Fuggle *Nuffield Dept of Surgery, John Radcliffe Hosp*
R Bicknell, A L Harris

Bladder cancer comprises two types - superficial and invasive. The prognosis is much worse for the latter group and the former often progress to become invasive. We have analysed mechanisms involved in angiogenesis in the earlier and later stages of bladder cancer. Vascular endothelial growth factor is upregulated in superficial tumours and is associated with a high recurrence rate. Assays are being developed to detect the secretion of this growth factor in the urine and relate it to prognosis. Heparin-like antagonists may be of value in preventing recurrence and invasion. Potential new intra-vesical therapies are being developed based on inhibition of angiogenesis.

PROFESSOR ADRIAN L HARRIS

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A Murine Model for Antibody-directed Targeting of Vascular Endothelial Cells in Solid Tumors[1]

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ABSTRACT

An attractive approach to the therapy of solid tumors would be to target cytotoxic agents or coagulants to the vasculature of the tumor rather than to the tumor cells themselves. This strategy has 3 advantages: (*a*) it should be applicable to many types of solid tumors because all require a blood supply for survival and growth; (*b*) the target endothelial cells are directly accessible through the blood and are normal cells, making the outgrowth of resistant mutants unlikely; and (*c*) there is an in-built amplification mechanism because thousands of tumor cells are reliant on each capillary for nutrients and oxygen. Despite its theoretical attractions, the approach of tumor vascular targeting has not been testable because antibodies that recognize tumor vascular endothelial cell antigens with adequate specificity are currently not available. In this study, we developed a model system in which to investigate the antibody-directed targeting of vascular endothelial cells in solid tumors in mice. A neuroblastoma transfected with the mouse interferon-γ gene, *C1300(Mur)*, was grown in antibiotic-treated BALB/c nude mice. The interferon-γ secreted by the tumor induces the expression of major histocompatability complex Class II antigens on the tumor vascular endothelium. Class II antigens are absent from the vasculature of normal tissues, although they are present on B-lymphocytes, cells of monocyte/macrophage lineage, and some epithelial cells. Anti-Class II antibody administered i.v. strongly stains the tumor vasculature, whereas an antitumor antibody directed against a major histocompatability complex Class I antigen of the tumor allograft produces classical perivascular tumor cell staining. This model should enable the theoretical superiority of tumor vascular targeting over conventional tumor cell targeting to be tested.

INTRODUCTION

In contrast with their efficacy in lymphomas (1, 2), monoclonal antibodies and immunoconjugates have proved relatively ineffective in the treatment of the major carcinomas (3, 4). The principal reason for this is that solid tumors are rather impermeable to antibody-sized molecules: specific uptake values of less than 0.001% injected dose/g of tumor are not uncommon in human studies (5, 6). Furthermore, antibodies that enter the tumor mass do not distribute evenly for 3 reasons. (*a*) The dense packing of tumor cells and fibrous tumor stroma present a formidable physical barrier to macromolecular transport and, combined with the absence of lymphatic drainage, create an elevated interstitial pressure in the tumor core that reduces extravasation and fluid convection (7, 8). (*b*) The distribution of blood vessels in most tumors is disorganized and heterogeneous, so that some tumor cells are separated from extravasating antibody by large diffusion distances (8). (*c*) All of the antibody entering the tumor may become adsorbed in perivascular regions by the first tumor cells encountered, leaving none to reach tumor cells at more distant sites (7, 9–11).

A solution to the problem of poor penetration of antibodies into solid tumors is to attack the endothelial cells lining the blood vessels in the tumor. This approach offers 3 advantages over direct targeting of tumor cells. (*a*) The target cells are directly accessible to i.v. administered therapeutic agents, permitting rapid localization of a high percentage of the injected dose (9). (*b*) Since each capillary provides oxygen and nutrients for thousands of cells in its surrounding "cord" of tumor, even limited damage to the tumor vasculature could produce an avalanche of tumor cell death (12, 13). (*c*) The outgrowth of mutant endothelial cells lacking the target antigen is unlikely because they are normal cells.

For tumor vascular targeting to succeed, antibodies are required that recognize tumor endothelial cells but not those in normal tissues. Although several antibodies have been raised (14–17), none has shown the required degree of specificity. However, numerous differences between tumor blood vessels and normal ones have been documented (reviewed in Refs. 12 and 18–20) that suggest that such antigenic differences exist. Tumors elaborate angiogenic factors (21) and cytokines (22, 23), which alter the behavior and phenotype of local endothelial cells. Vascular endothelial cells in tumors incorporate [^{3}H]-thymidine at a rate 30-fold greater than those in miscellaneous normal tissues (24), suggesting that proliferation-linked determinants could serve as markers for tumor vascular endothelial cells. Tumor angiogenesis requires detachment of endothelial cells from their underlying basement membrane before migration (21), so it is possible that antibodies to integrin extracellular matrix receptors may be selective for tumor endothelial cells because integrins are sequestered on the basal surfaces of normal quiescent endothelia (25, 26).

In this report we describe a murine model for antibody-directed targeting of tumor vascular endothelial cells. We show that an IFN[3]-γ-producing tumor growing in antibiotic-treated BALB/c *nu/nu* mice induces the expression of MHC Class II antigens on the tumor vascular endothelium. MHC Class II is absent from the vasculature of normal tissues, although it is present on B-lymphocytes, cells of monocyte/macrophage lineage, and some epithelia. Anti-Class II antibody administered i.v. localizes rapidly and strongly to the tumor vasculature, whereas an antitumor antibody, directed against a Class I antigen of the tumor allograft, produces classical perivascular tumor cell staining. This model should thus enable the theoretical superiority of tumor vascular targeting over conventional tumor cell targeting to be tested.

MATERIALS AND METHODS

Animals. BALB/c *nu/nu* mice were purchased from Simonsen (Gilroy, CA). All animals were maintained in microisolation units on

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[2] To whom requests for reprints should be addressed, at the Cancer Immunobiology Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8576.

[3] The abbreviations used are: IFN, interferon; BSA, bovine serum albumin; CD, cluster determinant; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; MEM, modified Eagle's medium; MHC, major histocompatibility complex; rIFN-γ, recombinant murine interferon-γ; NK, natural killer; PBS-BSA-N_3, phosphate-buffered saline containing 0.2% (w:v) bovine serum albumin and 0.2% (w:v) NaN_3; PBS-T, phosphate-buffered saline containing 0.05% (v/v) Tween 20 (Sigma).

sterilized food and water. Where indicated, tetracycline-HCl (Vedeo, St. Joseph, MO) was added to drinking water to a final concentration of 1.1 mg/ml (27). The strain carries the H-2^d haplotype.

Cells and Culture Conditions. All cell lines used in this study were cultured in MEM supplemented with 10% (v/v) fetal calf serum, 2.4 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10% CO_2. The C1300 neuroblastoma line was established from a spontaneous tumor that arose in an A/Jax mouse in 1940 (28). The C1300(Muγ)12 line, hereafter referred to as C1300 (Muγ), was derived by transfection of C1300 cells with the murine IFN-γ gene using the IFN-γ expression retrovirus pSVX (MuγδA_4) (29, 30), and was cultured in MEM as above containing 1 mg/ml G418 (Geneticin; Sigma). Both lines carry the MHC haplotype H-$2K^k$, I-A^k, I-E^k, D^d. C1300 and C1300(Muγ) cells were grown in regular tissue culture flasks or, when large quantities were required for *in vivo* experiments, in cell factories (Baxter, Grand Prairie, TX). Cells from s.c. tumors were recovered for *in vitro* analysis by gentle mincing in MEM. After tumor cells had adhered overnight, the monolayers were washed twice with MEM to remove nonadherent contaminant host cells. Tumor conditioned media were prepared by seeding C1300 and C1300(Muγ) cells at 25% of confluent density and culturing them for 4 days. Conditioned media were dialyzed for 16 h against MEM without FCS to remove G418, filtered through a 0.22-μM membrane, and stored at 4°C for no more than 1 week before assay. Aliquots of anti-IFN-γ antibodies (see "Monoclonal Antibodies") sufficient to neutralize 200 IU of murine IFN-γ/ml of conditioned medium were added to some samples 24 h before assay. The SVEC-10 murine endothelial cell line, hereafter referred to as SVEC, was kindly provided by Dr. M. Edidin, Department of Biology, The Johns Hopkins University, Baltimore, MD, and was derived by immortalization of lymph node endothelial cells from a C3H (H-2^k) mouse with SV40 (31). For some experiments, SVEC cells were cultured for 72 h with 100 IU/ml rIFN-γ (a generous gift from Dr. F. Balkwill, Imperial Cancer Research Fund, London, England) or tumor-conditioned medium. In addition, 200 IU/ml anti-IFN-γ antibody were added to some flasks at the beginning of the 72-h culture period.

Monoclonal Antibodies. The M5/114.15.2 (hereafter referred to as M5/114) and 11–4.1 hybridomas were purchased from the American Type Culture Collection (Rockville, MD) and were grown in MEM-10% FCS. The antibodies were purified from culture supernatant by precipitation in 50% ammonium sulfate and affinity chromatography on Protein A. The rat IgG2b antibody, M5/114, detects an Ia specificity on I-A^b, I-A^q, I-A^d I-E^d, and I-E^k molecules (32). Thus, the antibody recognizes I-E^k molecules on SVEC (H-2^k) cells and I-A^d and I-E^d, hereafter referred to collectively as Ia^d, on cells from BALB/c *nu/nu* mice (H-2^d haplotype). The anti-Ia^d reactivity of M5/114 was confirmed in this study by FACS analyses with the Ia^d-expressing B-lymphoma line, A20/25 (33). The mouse IgG2a antibody 11–4.1 recognizes H-$2K^k$ but not H-$2K^d$ molecules (34), and so binds to H-$2K^k$ on C1300 and C1300(Muγ) cells but is unreactive with MHC antigens from BALB/c *nu/nu* mice. Isotype-matched control antibodies of irrelevant specificity were CAMPATH-2 (rat IgG2b, anti-human CD7) (35) and WT-1 (mouse IgG2a, anti-human CD7) (36). Purified preparations of CAMPATH-2 and WT-1 were generous gifts from Dr. G. Hale (Department of Pathology, Cambridge University, Cambridge, England) and Dr. W. Tax (Sint Radboudzeikenhuis, Nijmegen, The Netherlands), respectively. Rat anti-mouse endothelial cell antibody MECA-20 (14) was donated as a concentrated culture supernatant by Dr. A. Duijvestijn (University of Limburg, The Netherlands) and used at a dilution of 1:200 for indirect immunoperoxidase staining. Rat antibodies against mouse macrophages (anti-Mac-1, M1/70) and mouse CD3 (KT 31.1) were generously provided by Dr. P. Beverley (Imperial Cancer Research Fund, London, England) and the rat IgM antibody CZ-1, which reacts with B-cells, CD8+ T-cells, and NK cells but is specific for NK cells in SCID mice (37), was kindly provided by Dr. R. M. Welsh (University of Massachusetts Medical Center, Worcester, MA). Hamster anti-mouse IFN-γ antibody 1222–00 (38), used for specific neutralization of IFN-γ *in vitro*, was purchased from Genzyme (Boston, MA). Anti-mouse IFN-γ antibodies, XMG1.2 and R46A2, used in IFN-γ ELISAs, were kindly provided by Dr. N. Street (University of Texas Southwestern Medical Center, Dallas, TX). Purified 11–4.1, WT-1, and XMG1.2 antibodies were biotinylated by incubation with a 12.5-fold molar excess of *N*-hydroxysuccinimidobiotin amidocaproate (Sigma) for 1 h at room temperature followed by dialysis against 2 changes of PBS.

ELISA for Murine IFN-γ. Sandwich ELISAs for murine IFN-γ were carried out as described previously (39). In brief, the wells of flexible polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were coated with 50 μl/well of a 2 μg/ml solution of capture anti-IFN-γ antibody, R46A2, in PBS for 2 h at room temperature. Nonspecific protein binding sites were blocked with 20% FCS in PBS for 15 min at 37°C. The plates were washed 3 times in PBS-T, and 25 μl/well control and experimental samples in MEM-10% FCS were added. After incubating for 1 h at 37°C, the wells were washed as before and 50 μl/well of a 1 μg/ml solution of biotinylated anti-IFN-γ antibody XMG1.2 in PBS-T containing 1% BSA were added. After incubation for 30 min at 37°C, the wells were washed as before and incubated with 75 μl of a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (DAKO) for 1 h at room temperature. After thorough washing in PBS-T, the wells were incubated for 30 min with 100 μl/well of a 1 mg/ml solution of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in citrate/phosphate butter containing 0.003% (v:v) H_2O_2. Reaction product was measured as A_{405}-A_{490}. IFN-γ levels in experimental samples were calculated by reference to a recombinant murine IFN-γ standard solution in MEM-10% FCS.

Indirect Immunofluorescence. SVEC, C1300, and C1300(Muγ) cells were prepared for FACS analyses as described previously (22). All manipulations were carried out at room temperature. In brief, 50 μl of a cell suspension at 2–3 × 10^6 cells/ml in PBS-BSA-N_3 were added to the wells of round-bottomed 96-well microtiter plates (Falcon 3910). Optimal dilutions of rat or mouse antibodies were distributed in 50-μl volumes, and the plates sealed. After 15 min, the cells were washed 4 times by centrifuging the plates at 800 × *g* for 30 s, removing the supernatants, and resuspending the cells in 150 μl/well PBS-BSA-N_3. Fluorescein isothiocyanate-conjugated rabbit antibodies against rat or mouse IgG (ICN, High Wycombe, England), diluted 1:20 in PBS-BSA-N_3, were distributed in 50-μl volumes into the appropriate wells. The cells were incubated for a further 15 min and washed as before. Cell-associated fluorescence was measured on a FACScan (Becton-Dickenson, Fullerton, CA). Data were analyzed using the CONSORT 30 program.

Preparation of Tissues and Immunohistochemistry. For the establishment of solid tumors, a total of 2 × 10^7 C1300 or C1300(Muγ) cells, or a mixture of the two, in 200 μl MEM-30% FCS was injected s.c. into the right anterior flank of BALB/c *nu/nu* mice. Tumor diameters were measured at regular intervals and the animals were euthanized after 16 days (rapidly growing tumors) or 20 days (slowly growing tumors). Tumors and normal tissues were excised immediately and snap-frozen over liquid nitrogen. Normal tissues were also harvested from non-tumor-bearing animals. Antibody localization experiments were performed in animals bearing 1 cm s.c. tumors induced by injection of C1300 and C1300(Muγ) in the ratio 7:3. One hundred μg of unconjugated M5/114 or CAMPATH-2 antibodies or 100 μg biotinylated 11–4.1 or WT-1 antibodies in 100 μl PBS were injected i.v. At various times thereafter, the animals were euthanized and their circulation was flushed with PBS for 5 min before removal and freezing of tumors and normal tissues as before. Eight-μM frozen sections were cut on a Tissuetek 2 cryostat (Baxter) and air-dried for 2 h at room temperature. Slides were stored at −20°C for up to 3 months before assay. Indirect immunoperoxidase staining for rat IgG was adapted from a method described previously (40). Briefly, sections were allowed to return to room temperature, air dried for 30 min, and fixed in acetone for 15 min. After rehydration in PBS for 5 min, sections were incubated in a humidified chamber for 45–60 min with primary antibodies, diluted optimally in PBS-0.2% BSA. After 2 washes in PBS, the sections were incubated for 30–45 min with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Carpinteria, CA) diluted 1:10 in PBS-0.2% BSA supplemented with 20% normal mouse serum (ICN) to block antibodies cross-reacting with mouse immunoglobulins. After a further 2 washes in PBS, the reaction product was developed using 0.5 mg/ml

3',3'-diaminobenzidine (Sigma) containing 0.01% (v/v) hydrogen peroxide for 8 min. The sections were counterstained with Mayer's hematoxylin (Sigma) for 15 s, dehydrated in absolute ethanol, cleared in xylene, and mounted with Accumount 60 medium (Baxter). Indirect immunoperoxidase staining with biotinylated mouse antibodies was carried out in the same manner, except that peroxidase-conjugated streptavidin-biotin complex, diluted 1:50 in PBS with no blocking serum, was used as the second layer.

RESULTS

Murine IFN-γ Levels in C1300(Muγ)-conditioned Medium. C1300(Muγ)-conditioned medium contained 50.2–63.5 IU/ml murine IFN-γ, in accordance with previous reports (30). By contrast, less than 5 IU/ml IFN-γ were detected in C1300-conditioned medium or C1300(Muγ)-conditioned medium to which an excess of neutralizing anti-IFN-γ antibody had been added 24 h before assay.

Induction of MHC Class II (I-E^k) on SVEC Cells by rIFN-γ in C1300(Muγ)-conditioned Medium. As shown in Fig. 1*a*, unstimulated SVEC cells did not express I-E^k. By contrast, a large majority of cells preincubated with rIFN-γ (Fig. 1*a*) or with C1300(Muγ)-conditioned medium (Fig. 1*b*) expressed significant levels of I-E^k, and this induction was almost completely blocked by anti-IFN-γ. Treatment of SVEC cells with rIFN-γ or C1300(Muγ)-conditioned medium did not cause nonspecific antibody binding since the isotype-matched control antibody did not bind to the cells. These results were confirmed by indirect immunoperoxidase staining of cytospin preparations (data not shown).

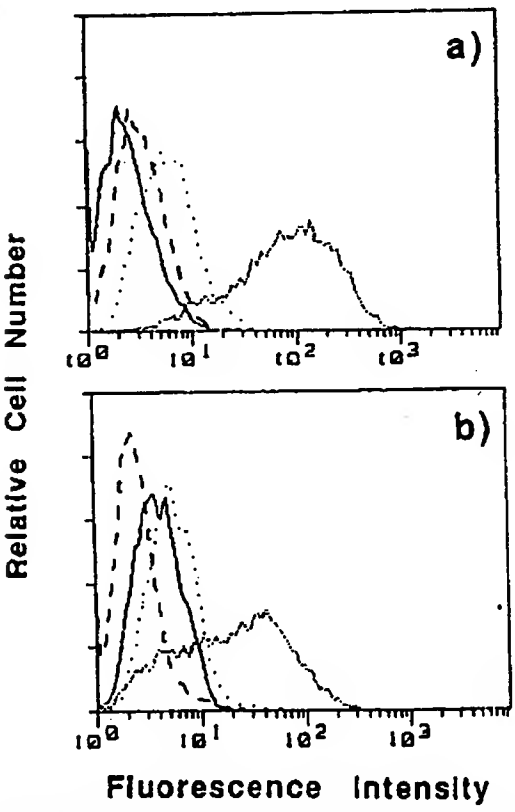

Fig. 1. Induction of I-E^k on SVEC cells by IFN-γ in C1300(Muγ)-conditioned medium. *a*, SVEC cells were cultured for 72 h in regular medium (—), rIFN-γ (. . . .), or rIFN-γ plus excess neutralizing anti-IFN-γ antibody (. . . .). Their expression of I-E^k was then measured by M5/114 antibody binding by indirect immunofluorescence using the FACS. Other cultures were treated with rIFN-γ and stained with an isotype-matched control antibody (- - - -). *b*, SVEC cells were cultured for 72 h in C1300-conditioned medium (—), C1300(Muγ)-conditioned medium (. . . .), or C1300(Muγ)-conditioned medium plus excess neutralizing anti-IFN-γ antibody (. . . .). Their expression of I-E^k was then measured as in *a*. Other cultures were treated with C1300(Muγ)-conditioned medium and stained with an isotype-matched control antibody (- - - -).

These findings suggested that vascular endothelial cells in tumors containing sufficient quantities of IFN-γ-secreting C1300(Muγ) cells should be induced to express high cell surface levels of MHC Class II molecules.

Expression of MHC Class I (H-2K^k) and Class II (I-E^k) by C1300 and C1300(Mnγ) Cells. Since IFN-γ can induce MHC Class II antigen expression in diverse cell types (41–43) and since the M5/114 antibody cross-reacts with I-E^k, we determined whether the M5/114 antibody, intended for use to target tumor endothelial cells *in vivo*, would also (Fig. 2*a*) bind to the tumor cells themselves. As shown in Fig. 2*a*, C1300(Muγ) cells expressed I-E^k, but at levels 10–20-fold lower than those on SVEC cells stimulated with IFN-γ.

Similarly, C1300 cells expressed detectable but low levels of H-2K^k, whereas C1300(Muγ) cells displayed uniformly high levels, approximately 20-fold greater than on the parental line (Fig. 2*b*). This result was expected from the known autocrine Class I-inducing activity of IFN-γ and is in keeping with a previous report (30). Coculture of C1300(Muγ) cells and C1300 cells induced homogeneous expression of I-E^k and H-2K^k on both populations (Fig. 2). Induction of these antigens on C1300 cells appears to be caused by IFN-γ released into the culture medium by the C1300(Muγ) cells since the effect was neutralized by anti-IFN-γ antibodies (30).

Growth of C1300 and C1300 (Muγ) Tumors in Immunodeficient Mice and Induction of Ia^d on Tumor Vascular Endothelial Cells. We first attempted to grow s.c. C1300(Muγ) tumors in BALB/c *nu/nu* mice because the strain carries the MHC haplotype (H-2^d) with which the anti-MHC Class II antibody M5/114 reacts, and because they would not be expected to reject the tumors, as do syngeneic immunocompetent A/J animals (30). For unknown reasons, inocula composed entirely of C1300-(Muγ) cells failed to produce progressively growing tumors in BALB/c *nu/nu* mice. Conversely, pure C1300 inocula displayed 100% tumorigenicity but, as expected, did not contain Ia^d-positive endothelial cells. To identify a combination that would yield a high percentage of tumor takes and reliable growth kinetics, and cause Ia^d induction of a large majority of intratumoral endothelial cells, several ratios of C1300 and C1300-(Muγ) cells were inoculated into BALB/c *nu/nu* mice. As shown in Fig. 3, mixtures containing C1300 and C1300(Muγ) cells in the ratio 9:1 produced rapidly growing tumors but, when sections of the tumors were stained with anti-Ia^d antibody by the indirect immunoperoxidase technique, none of the endothelial cells in the tumor was found to be stained. Dropping the ratio of C1300:C1300(Muγ) to 8:2 gave rapidly growing tumors in which approximately 50% of blood vessels were Ia^d-positive. Dropping the ratio further to 7:3 or 5:5 produced tumors that grew quite rapidly and contained a large majority of Ia^d-positive vessels. Dropping the ratio still further to 3:7 produced tumors in no more than half of the animals, and those tumors that became palpable failed to grow beyond 6 mm in diameter. Histological analyses of the latter revealed no morphologically recognizable intact blood vessels and, hence, it was not possible to ascertain their level of Ia^d expression.

Of the 2 usable C1300:C1300(Muγ) ratios identified, 7:3 and 5:5, the ratio of 7:3 was adopted for the remainder of this study because the take rate was higher (100% *versus* 80%) and the

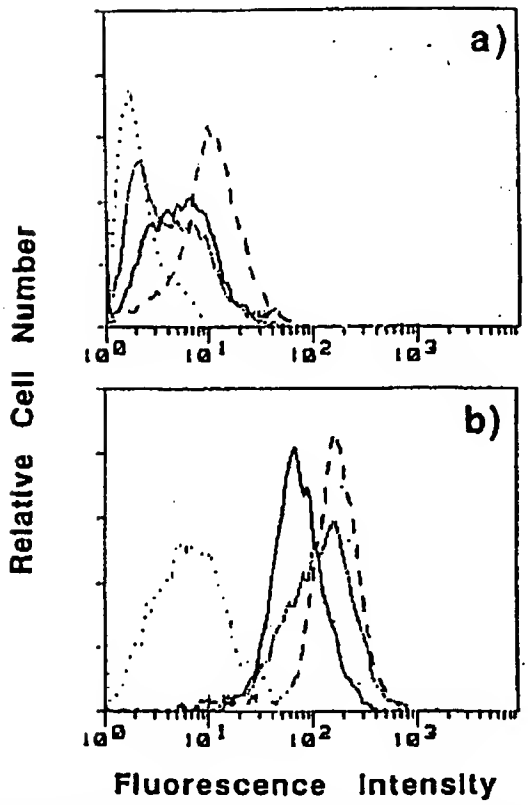

Fig. 2. Expression of I-E^k and H-2K^k by pure and mixed populations of C1300 and C1300(Muγ) cells. C1300 cells (. . . .), C1300(Muγ) cells (- - - -), a mixture of C1300 and C1300(Muγ) cells in the ratio 7:3 cocultured *in vitro* (. . . .), or cells recovered from a mixed s.c. tumor in a BALB/c *nu/nu* mouse (——) were stained with (*a*) anti-I-E^k antibody or (*b*) anti-H-2K^k antibody by indirect immunofluorescence using the FACS. No staining of any tumor cell population was seen with the isotype-matched control antibodies.

variability in tumor growth rate between individual animals was lower (data not shown).

Distribution of Ia^d in BALB/c Nude Mice. The distribution of M5/114 binding in tissues from tumor-bearing BALB/c *nu/nu* mice is shown in Table 1. In s.c. tumors, most or all vascular endothelial cells and numerous interstitial macrophages were stained (Fig. 4). In most organs, the binding of M5/114 reflected the classical distribution of MHC Class II antigens, being restricted to B-cells in lymphoid organs, resident macrophages in all tissues studied except brain, tissue-specific elements of the reticuloendothelial system, such as liver Kupffer cells and Langerhans cells of the skin, and to a minor subpopulation (5–8%) of bone marrow cells. In addition, staining was occasionally seen in some kidney tubules. When sections of small and large intestine from BALB/c *nu/nu* mice were examined, heavy labeling of both epithelial and endothelial cells was seen in both regions. The staining of *nu/nu* mouse intestine was found to be related to the microbiological status of the animals and is discussed below. Apart from in the gut, no staining of endothelial cells with M5/114 was seen in any tissues examined. The distribution of Ia^d antigens in normal tissues was not affected by the presence of the tumor because the staining pattern of M5/114 was identical in non-tumor-bearing mice.

Attenuation of Ia^d Expression on Intestinal Endothelium and Epithelium of Nude Mice by Administration of Antibiotics. In BALB/c *nu/nu* mice, most epithelial cells from all regions of the gut were intensely stained with anti-Ia^d antibody. In addition, some endothelial cells in both upper and lower bowel bound M5/114 antibody, particularly those associated with villi of the small intestine (Fig. 5*a*). When the animals were treated with p.o. tetracycline-HCl, a broad-spectrum antibiotic, for 1–3 weeks there was a progressive dimunition of Ia^d expression in the ileum and elsewhere in the gut, so that binding of M5/114 was in most sections restricted to the luminal membranes of a minority of epithelial cells (Fig. 5*b*). Light cytoplasmic staining of occasional endothelial cells was observed in some antibiotic-treated animals. The pattern of epithelial and endothelial Ia^d expression was not homogeneous and the intensity of M5/114 staining correlated with the frequency of CD3+ T-lymphocytes in the adjacent epithelium, subepithelium, and lamina propria (Fig. 5*c*). Antibiotic treatment was associated with a dramatic decrease in the numbers of intravillous CD3-positive cells: after 3 weeks, practically all had disappeared from all parts of the villi (Fig. 5*d*) and associated lymphoid deposits, and there was a coincident decline in Ia^d expression on surrounding epithelial and endothelial cells. The majority of intravillous T-cells were CD8+ CD4- (data not shown).

Specific Localization of i.v. Administered Anti-Ia^d Antibody to Tumor Vasculature, B-Cells, and Macrophages in Antibiotic-treated Nude Mice. Tumor-bearing BALB/c *nu/nu* mice were given i.v. injections of anti-Ia^d or the isotype-matched control antibody and euthanized 1, 4, or 24 h later. The *in vivo* localization of anti-Ia^d antibody in tumor and normal tissues is shown in Table 1 and Fig. 6. Anti-Ia^d antibody was found on the luminal membrane and in the cytoplasm 6b) of most or all tumor vascular endothelial cells 1 h after injection (Fig. 6, *a* and *b*). A similar pattern was seen at 4 h after injection, but by 24 h the labeling of tumor endothelial cells was weaker and entirely intracellular, consistent with the progressive internalization and metabolism of the antibody by endothelial cells (Table 1). Also, at 24 h small amounts of antibody were detectable in the immediate perivascular regions of the tumor.

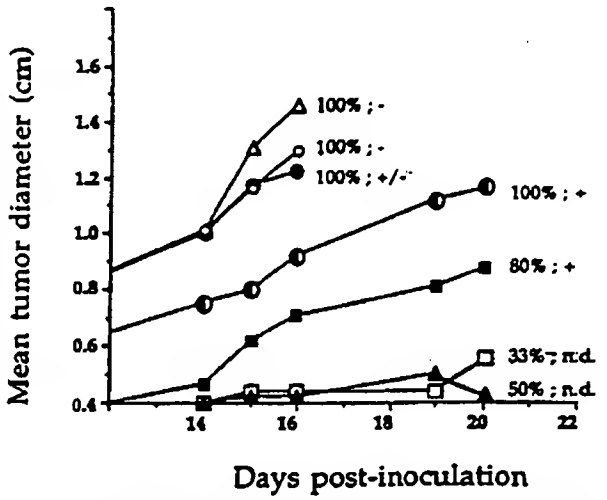

Fig. 3. Tumorigenicity, growth, and tumor endothelial cell Ia^d expression in pure and mixed s.c. C1300 and C1300(Muγ) tumors. BALB/c *nu/nu* mice were given injections of a total of 2×10^7 tumor cells in which the ratios of C1300:C1300(Muγ) cells were 10:0 (Δ), 9:1 (○), 8:2 (●), 7:3 (◐), 5:5 (▥), 3:7 (▲), or 0:10 (□). Vertical axis, mean diameter of the tumors at various times after injection. Also shown are the percentage of animals in each group that developed tumors. The proportion of Ia^d-positive vascular endothelial cells was categorized as follows: +, 75–100%; +/−, 25–75%; −, 0–5%; n.d., not determined because no intact blood vessels were visible. SD were <15% of mean diameters and are not shown.

Table 1 *Localization of i.v. administered anti-Ia^d antibody in C1300(Muγ) tumor-bearing mice*[a]

Tissue	Antigen expression	Localization in vivo		
		1 h	4 h	24 h
Tumor[b]	EC,[c] Mϕ	EC[d]	EC	EC[e]
Brain	None	None	None	None
Colon[a]	Some crypt epithelium and EC, Mϕ	None	None	None
Ileum[a]	Some villous epithelium and EC, Mϕ	None	None	None
Heart	Interstitial Mϕ	None	None	None
Kidney	Occasional tubule epithelium, Mϕ	None	None	None
Liver	KC, numerous Mϕ in parenchyma	KC[d]	KC	KC,[e] some Mϕ
Lung	Numerous Mϕ in parenchyma	None	None	None
Pancreas	Numerous Mϕ in parenchyma	None	None	None
Skin[f]	Langerhans cells	None	None	None
Spleen	RP Mϕ, MZ Mϕ, MZ B cells, some lymphocytes in WP	MZ	MZ, WP	MZ, RP, WP
Bone marrow	5–8% of cells	ND	ND	<5% of cells

[a] Experiments performed with antibiotic-treated BALB/c *nu/nu* mice.
[b] Mixed tumor of 7:3 C1300:C1300(Muγ) cells grown s.c.
[c] EC, endothelial cells; KC, Kupffer cells; Mϕ, macrophages; MZ, marginal zone; RP, red pulp; WP, white pulp; ND, not determined.
[d] Strong staining, including discernable labeling of luminal membranes.
[e] Weaker staining, entirely intracellular.
[f] Either adjacent to, or distant from tumor.

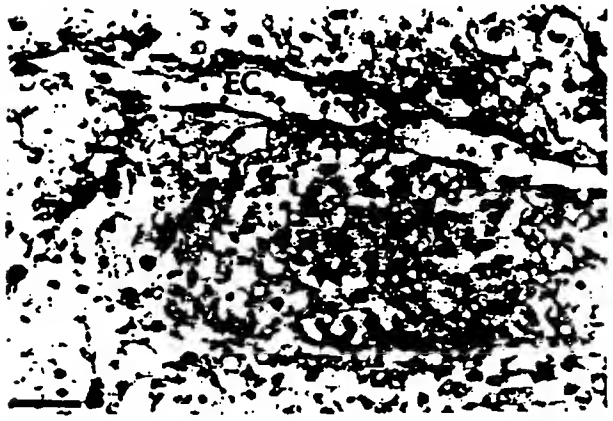

Fig. 4. Expression of Ia^d on vascular endothelial cells and macrophages in mixed C1300:C1300(Muγ) tumors. Frozen sections of the tumor were stained with anti-Ia^d antibody, M5/114, using the indirect immunoperoxidase technique. Capillary endothelial cells (EC) and interstitial macrophages (M) are intensely stained. *bar*, 20 μm.

Anti-Ia^d antibody was bound to Kupffer cells in the intravascular compartment of the liver within 1 h of injection (Fig. 6*c*). At later times after injection, internalization and degradation of the antibody were apparent (Table 1). Adjacent sinusoidal endothelial cells were not stained (Fig. 6*c*). The high permeability of hepatic fenestrated endothelia was indicated by the penetrance of the antibody to reach some hepatic parenchymal macrophages (Table 1). In the spleen, perivascular B-cells and macrophages in white pulp marginal zones were stained within 1 h, showing that the vasculature of this organ was particularly permeable to antibody (Fig. 6*d*). At later stages, the antibody penetrated throughout the splenic lymphoid compartment and also labeled a minority of red pulp macrophages (Table 1). In organs other than the liver and spleen, macrophages and related cells such as the Langerhans cells of the skin were unstained probably because their vascular endothelium contains tight junctions and is relatively impermeable to antibodies. After 24 h, a small percentage (<5%) of bone marrow cells were labeled (Table 1).

Anti-Ia^d antibody was bound to some endothelial cells in the ileum, duodenum, and colon of BALB/c *nu/nu* mice, but not elsewhere in the intestine, 1 h after injection. Antibiotic treatment for 1–3 weeks before injection of anti-Ia^d antibody completely abolished localization to gut endothelial cells. The isotype-matched control antibody was not detected in tumor or normal tissues at any time after injection.

Taken together, these results strongly indicate that, when injected into appropriate tumor-bearing animals anti-Ia^d antibody or immunoconjugates will localize effectively to most or all tumor endothelial cells while sparing life-sustaining normal tissues.

Perivascular Staining of Tumor Cells in Mice Given Injections of Antitumor (H-2K^k) Antibody. When frozen sections of s.c. tumors deriving from inocula of mixed C1300 and C1300(Muγ) cells (7:3) were stained with biotinylated anti-H-2K^k antibody, a homogeneous staining pattern was obtained (Fig. 7*a*). The levels of IFN-γ secreted by the C1300(Muγ) cells in the tumor were therefore sufficient to induce increased H-2K^k expression by the C1300 component of the tumor, in accordance with the *in vitro* co-culture experiments described above. The staining was specific because no staining was seen with the isotype-matched control antibody. No specific labeling of any normal tissue by anti-H-2K^k antibody was found, as expected since this antibody was raised in an H-2^d mouse strain.

In contrast with the rapid binding of i.v.-administered anti-Ia^d antibody to tumor vasculature, no significant accumulation of anti-H-2K^k antibody was apparent 1 h after injection. After 4 h, however, anti-H-2K^k antibody was detected in small islands of tumor cells surrounding central capillaries (Fig. 7*b*). After 24 h, the antibody was bound to larger discrete areas of tumor cells, but staining intensity was diminished relative to the earlier time points. Even with localization times of up to 72 h, homogeneous labeling of all tumor cells was not achieved (data not shown).

No localization of anti-H-2K^k antibody was found in any normal tissues, and binding of the isotype-matched control antibody was not detectable in tumor or normal tissues.

DISCUSSION

This report describes a murine model for studying the antibody-directed targeting of vascular endothelial cells in solid tumors. In this model, IFN-γ gene-transfected tumor cells growing in antibiotic-treated nude mice release IFN-γ, which induces the *de novo* expression of MHC Class II antigens on the tumor vasculature. MHC Class II is absent from the vasculature in the normal tissues of these mice and hence the Class II induced on the tumor vascular endothelial cells serves as a specific marker. Class II is present on B-lymphocytes, Kupffer cells, and other cells of monocyte/macrophage lineage, but

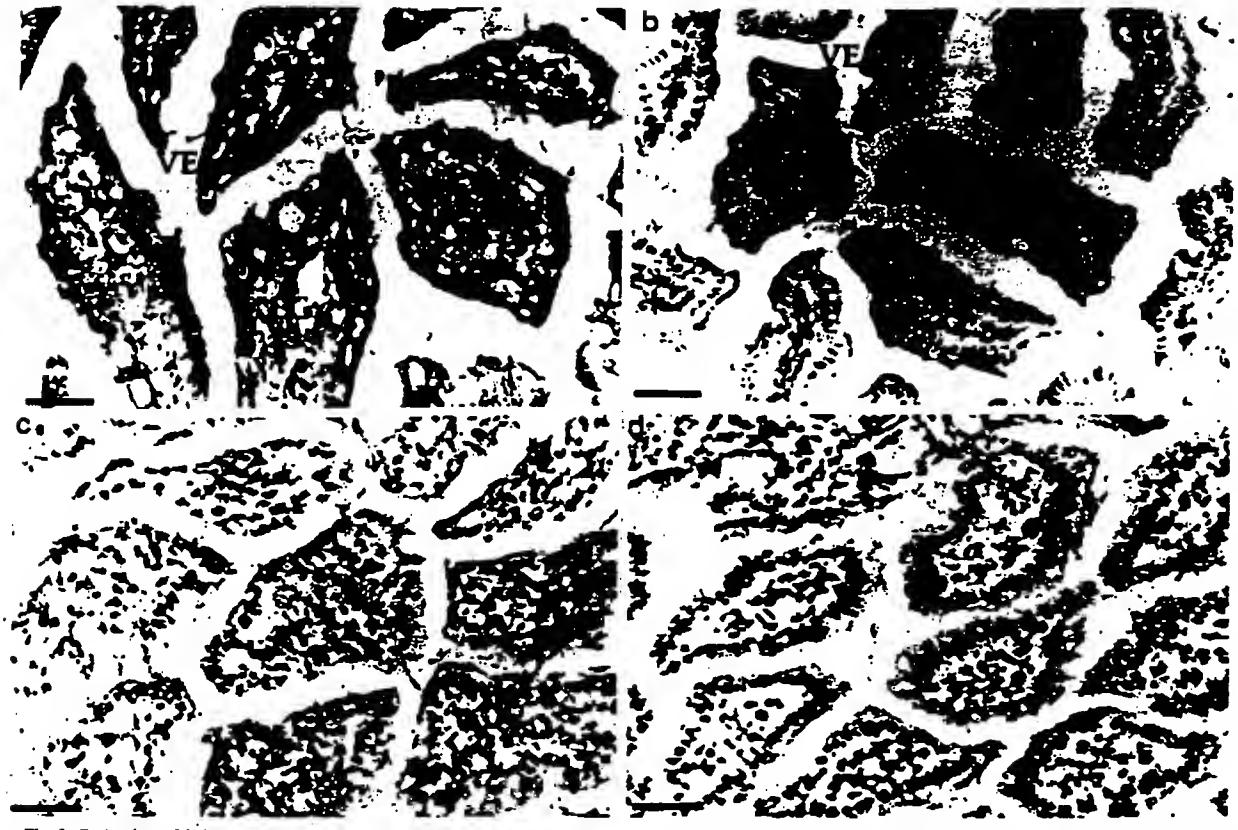

Fig. 5. Reduction of Ia^d expression and CD3+ cell infiltration in the colon of antibiotic-treated mice. Frozen sections of colon from untreated (*a* and *c*) and antibiotic-treated (*b* and *d*) BALB/c *nu/nu* mice were stained with anti-Ia^d antibody, M5/114 (*a* and *b*), or anti-CD3 antibody (*c* and *d*). In untreated mice (*a*), there is heavy labeling of Ia^d on intravillous capillaries (EC) and villous epithelium (VE), whereas in antibiotic-treated mice (*b*) there is light staining of Ia^d on some epithelial cells (VE) but capillaries (EC) are unstained. Similarly, in untreated mice (*c*) numerous small round CD3+ cells are visible in villous connective tissue (*arrowheads*), whereas in antibiotic-treated mice (*d*) only occasional epithelium and CD3+ cells are seen (*arrowheads*). *Bars*, 20 μM.

these cells are not life-sustaining so their temporary absence after targeting with cytotoxic immunoconjugates should be tolerable. IFN-γ also induces the tumor cells themselves to express high levels of the MHC Class I antigen, H-$2K^k$, which can serve as a tumor cell-specific marker in BALB/c *nu/nu* mice, which carry the H-$2K^d$ haplotype. Thus, anti-Ia^d and anti-H-$2K^k$ antibodies injected systemically localize selectively to tumor vascular endothelial cells and tumor cells, respectively, which enables the approaches of targeting the tumor vasculature and the tumor cells to be compared in this model, or used in combination.

It was necessary to dilute the C1300(Muγ) cells with C1300 parental cells in the ratio 3:7 to establish progressively growing s.c. tumors in which the vascular endothelial cells were Class II (Ia^d)-positive. Undiluted C1300(Muγ) cells were poorly tumorigenic in BALB/c *nu/nu* mice, in contrast with a prior report (30). Vascular dysfunction appeared to be the reason why pure C1300(Muγ) tumors would not grow beyond a diameter of 5–6 mm. Staining of sections of tumors with the anti-endothelial cell antibody MECA 20 revealed that the vessels were morphologically atypical with no visible lumens. It is possible that excessively high intratumoral IFN-γ levels in pure C1300(Muγ) tumors caused direct vascular toxicity or activated macrophages in the tumor to become cytotoxic for endothelial cells (44), although the number, location, and activation status of Mac-1+ macrophages and CZ-1+ NK cells did not vary significantly between C1300, C1300(Muγ), and mixed tumors.

Anti-Ia^d antibody injected i.v. bound rapidly and homogeneously to vascular endothelial cells in the tumor, confirming the immediate accessibility of intravascular targets (9). Remarkably, the inductive influence of IFN-γ from C1300(Muγ) cells was completely restricted to the tumor mass: endothelial cells in the overlying area of skin expressed no detectable Ia^d and did not bind any i.v.-injected anti-Ia^d antibody. It is likely that IFN-γ entering the systemic circulation is neutralized by a specific binding protein, perhaps a soluble form of the IFN-γ receptor (45), whose normal role may be to down-regulate cytokine activity (46) or to restrict it to the immediate locale of secretion.

Ia^d antigens are not restricted solely to tumor endothelial cells. MHC Class II antigens are expressed constitutively by B-cells, activated T-cells, and cells of the monocyte/macrophage lineage in humans and rodents (47, 48) and were found in this study also to be present on occasional proximal tubules in the kidney and on some epithelial cells in the intestine of antibiotic-treated BALB/c *nu/nu* mice. However, when injected i.v., only the hepatic Kupffer cells, splenic B-cells, and macrophages in the liver and spleen bound detectable amounts of the anti-Ia^d antibody: the potentially life-sustaining Class II-positive renal and gut epithelial cells were unstained. Localization of i.v.-injected anti-Ia^d antibody to hepatic Kupffer cells and splenic marginal zone B-cells occurred within 1 h, in accordance with the report of Kennel *et al.* (9). Presumably, the extreme permeability of the discontinuous splenic endothelium permits rapid

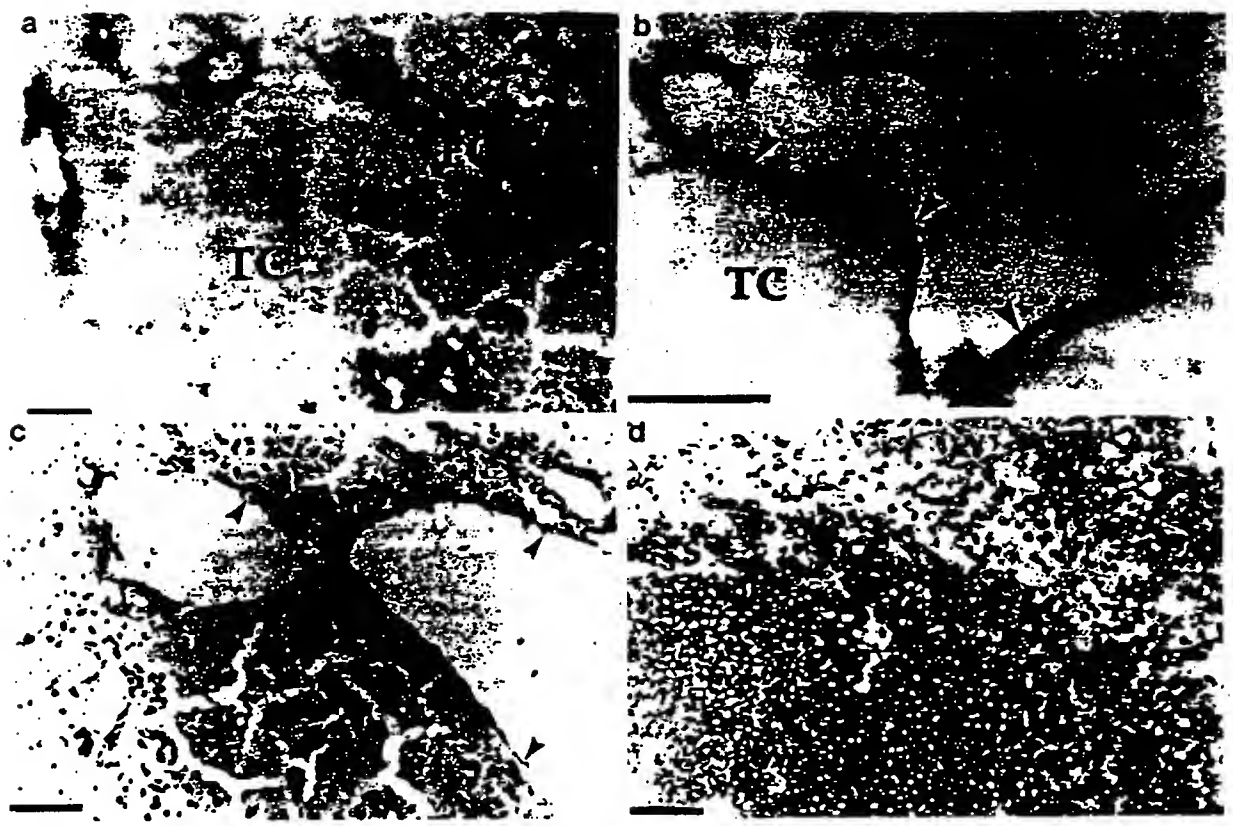

Fig. 6. Specific localization of i.v.-administered anti-Ia^d antibody in nude mice. Anti-Ia^d antibody, M5/114, was injected and 1 h later the tumor, liver, spleen, and colon were removed. Frozen sections were then stained for rat IgG using the indirect immunoperoxidase technique. In the tumor (*a* and *b*), all capillary endothelial cells (EC) are heavily labeled, but macrophages (M) and tumor cells (TC) are unlabeled. The localized anti-Ia^d antibody is particularly concentrated on the luminal membranes of tumor endothelial cells (*b, arrowheads*). *c*, in the liver, Kupffer cells (K) in sinusoidal crypts are stained but adjacent sinusoidal endothelial cells (*arrowheads*) are unstained. *d*, in the spleen, B-cells and macrophages adjacent to marginal zone sinusoids (S) are heavily labeled and there is some penetration of antibody deeper into the white pulp (WP). *Bars*, 10 μM (*a*), 20 μM (*b-d*).

extravasation of antibodies into the parenchyma of this organ and staining of the marginal zone B-cells (9). The reason for the lack of staining of renal and gut epithelial cells is probably that these cells are not readily accessible to i.v.-administered antibody because the antibody would have to diffuse across basement membranes and several tissue layers to reach these cells. In addition, it is likely that all the remaining anti-Ia^d antibody in the circulation was absorbed by more accessible splenic white pulp lymphocytes before significant extravasation into the red pulp (9, 11) or other normal tissues could occur. This is important because it illustrates a potentially critical pharmacokinetic difference between vascular targeting and tumor cell targeting. Because the tumor endothelial cells are so accessible to i.v.-administered antibody, the presence of a large "sink" of competing antigen in the blood or lymphoid organs should not prevent the antibody from reaching the target cells but should protect antigen-positive cells in most extravascular compartments. It is conceivable that an antibody recognizing a tumor vascular endothelial cell antigen that is shared by epithelial cells, for instance, might be targeted without the toxic side effects that have complicated therapy with anti-tumor cell immunoconjugates (49). Furthermore, even in the absence of such a sink, it is possible that operative specificity for tumor endothelial cells could be achieved in the face of cross-reactivity with extravascular normal tissues by decreasing the dose or by using rapidly cleared antibody fragments in the construction of the immunoconjugate. Anti-Ia^d antibody did localize to a small population of bone marrow cells, which were probably late stage myeloid progenitors. However, destruction of this population was not permanent or life threatening since no late occurring toxicity was seen in any animals and, 20 days after treatment with an anti-Ia^d immunotoxin, bone marrow aspirates contained unchanged numbers of granulocytes, monocytes/macrophages, and Ia^d+ cells.[4]

Although anti-Ia^d antibody did not localize to life-sustaining Ia^d+ extravascular tissues such as kidney tubules and gut epithelium, it did bind to gut endothelial cells in non-antibiotic-treated BALB/c *nu/nu* mice. These cells were as accessible as tumor endothelial cells and were required for survival since some regular BALB/c mice treated with high doses of M5/114 immunotoxins died from intestinal damage.[4] Murine endothelial cells do not express MHC Class II antigens *in vitro* (31, 50) or *in vivo* (51) unless stimulated with IFN-γ, so it is likely that induction of Ia^d on intestinal endothelial and epithelial cells was a result of local secretion of IFN-γ by T-cells (38) or activated NK cells (52, 53) in response to gut flora. In accordance with this view, numerous CD3+, CD8+ T-cells were observed in the villous stroma and epithelium of BALB/c *nu/nu* animals and their frequency correlated with the intensity of staining of

[4] F. J. Burrows, J. P. Overholser, and P. E. Thorpe, manuscript in preparation.

Fig. 7. Expression of H-2K^k and localization of i.v.-administered anti-H-2K^k antibody to perivascular regions in mixed C1300:C1300(Muγ) tumors. *a*, frozen sections of the tumor were stained with biotinylated anti-H-2K^k antibody, 11-4.1, using the streptavidin-biotin complex-horseradish peroxidase technique. Tumor cells show homogeneous staining by the anti-H-2K^k antibody. *Bars*, 20 μM. *b*, biotinylated anti-H-2K^k antibody was injected into a tumor-bearing BALB/c *nu/nu* mouse and 4 h later the tumor was removed. Frozen sections were stained with streptavidin-biotin complex-horseradish peroxidase. Tumor cells in the center of the field are heavily stained but others appear to have bound little or no antibody (*arrowheads*). *Bars*, 20 μM.

endothelial and epithelial cells with anti-Ia^d antibody. Furthermore, p.o. administration of tetracycline-HCl (a broad spectrum antibiotic) reversed T-cell infiltration, diminished Ia^d expression, and abolished localization of i.v.-injected anti-Ia^d antibody to gut endothelial cells. Antibiotic treatment had no effect on Ia^d expression by tumor endothelial cells. Most of the intravillous CD3+ cells observed in regular BALB/c *nu/nu* ileum were located within or immediately beneath the epithelial cell layer and had the phenotype CD4-CD8+, so it is most likely that they were thymus-independent T-cell receptor-$\gamma\delta$+ intraepithelial lymphocytes, which are present in BALB/c *nu/nu* mice (54), migrate in response to normal gut microbial colonization (54), and secrete IFN-γ (55).

Consistent with the findings of others (7, 9, 56, 57), an antitumor antibody directed against the H-2K^k antigen on C1300 and C1300(Muγ) cells showed perivascular staining of tumor cells after i.v. administration. In view of the homogeneous expression of H-2K^k by tumor cells *in vitro* and in sections of s.c. tumors, it is likely that the uneven intratumoral distribution of i.v.-injected anti-H-2K^k antibody was related to the vascular and interstitial physiology of the tumor (8, 11). This nicely demonstrates, in a single system, the limitations of using antitumor antibodies for targeting and the virtues of tumor vascular targeting. It may be possible to combine both approaches to advantage because the tumor cells that survive destruction of intratumoral blood vessels are likely to be those at the periphery of the tumor mass, close to the tumor-host interface. These areas are likely to be well vascularized by capillaries in adjacent normal tissues and have low interstitial pressure (8), so the surviving cells should be amenable to attack by antitumor immunoconjugates.

In summary, we describe a murine model with which to test the feasibility of targeting the vasculature of solid tumors. The model permits the antitumor effects of immunoconjugates directed against tumor vasculature to be compared with those of immunoconjugates directed against the tumor cells themselves. A forthcoming report will describe the effects of immunotoxins on large solid tumors in this model system.

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Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature

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ABSTRACT **Antibody-based therapy of solid tumors has met with limited success, chiefly because solid tumors are relatively impermeable to macromolecules. This problem could be circumvented by attacking the readily accessible endothelial cells of the tumor vascular bed. We have developed a model to test this "vascular targeting" approach in which cytokine gene transfection of the tumor cells causes them to induce an experimental marker selectively on tumor vascular endothelium. An anti-tumor endothelial cell immunotoxin caused complete occlusion of the tumor vasculature and dramatic regressions of large solid tumors. By contrast, a conventional anti-tumor cell immunotoxin of equivalent *in vitro* potency produced only minor, transient antitumor effects but, when combined, the two immunotoxins induced permanent complete remissions in over half of the animals. These experiments indicate that immunotoxins directed against recently described markers on vascular endothelial cells in human tumors could provide a general treatment for solid tumors in humans.**

Immunotoxins constructed by using chemical (1) or molecular (2) approaches have proved effective against lymphomas and leukemias (3) but thus far clinical results in carcinoma and melanoma patients have been disappointing (2, 4). The principal reason for this is that immunotoxins and other antibody conjugates permeate poorly and unevenly into solid tumors. Typically, only 0.001–0.01% of the injected dose of an antibody localizes to each gram of tumor in humans (5). The poor penetration is due to several interrelated factors. First, dense packing of tumor cells and the fibrous tumor stroma present a formidable physical barrier to macromolecular transport. Second, elevated interstitial pressure in the tumor core hinders extravasation and fluid convection (6, 7). Third, the antibody entering the tumor tends to become specifically adsorbed in perivascular regions by the first tumor cells encountered, leaving none to reach tumor cells at more distant sites (8).

A solution to the problem of poor penetration of antibodies into solid tumors would be to attack the endothelial cells lining the blood vessels of the tumor rather than the tumor cells themselves. The vascular endothelial cells are directly accessible to circulating therapeutic agents, and, since thousands of tumor cells are reliant on each capillary for oxygen and nutrients, even limited damage to the tumor vasculature should produce an avalanche of tumor cell death (9). In addition, the approach should be applicable to numerous types of solid tumors because all rely on their vasculature for growth (10). Despite its appeal, the "vascular targeting" approach has not hitherto been testable because antibodies with adequate specificity for tumor vascular endothelial cells have not been available (9). The recent discovery of markers of tumor endothelial cells in human cancers (11, 12) accentuates the need for animal models of vascular targeting with which to study the efficacy and pharmacology of this therapeutic strategy.

We have exploited the ability of cytokines to activate specific genes in vascular endothelial cells in order to develop a murine model to test the concept of antibody-directed targeting of tumor vasculature (13). A neuroblastoma cell line was transfected with the murine interferon γ (IFN-γ) gene by using a retroviral vector (14). When the transfectant C1300(Muγ) cells are grown subcutaneously in BALB/c *nu/nu* mice, they secrete IFN-γ, which activates capillary and venular endothelial cells within the tumor mass to express class II antigens of the major histocompatibility complex (MHC). Vascular endothelial cells in normal mouse tissues do not express MHC class II antigens unless activated by IFN-γ (15, 16), although MHC class II antigens are constitutively expressed by B cells, macrophages, and some epithelial cells (13). Similarly, a class II-negative murine endothelial cell line was induced to express class II antigens by activation with recombinant murine IFN-γ or C1300(Muγ) tumor-conditioned medium *in vitro* (13). When an anti-class II antibody was injected intravenously into C1300(Muγ) tumor-bearing mice, it localized within 1 hr to all tumor vascular endothelial cells. By contrast, a tumor-specific antibody directed against the unique MHC class I antigen of the tumor allograft, which strongly stained all C1300(Muγ) cells by fluorescence-activated cell sorting and in tissue sections of subcutaneous tumors, accumulated slowly and was restricted to perivascular regions of the tumor even 48 hr after injection (13). In this report, we describe the antitumor effects of ricin A-chain immunotoxins (17), prepared with anti-class II and anti-class I antibodies, in mice bearing large solid C1300(Muγ) tumors.

MATERIALS AND METHODS

Animals. BALB/c *nu/nu* mice were purchased from Simonsen Laboratories (Gilroy, CA). All animals were maintained in microisolation units on sterilized food and water. Tetracycline HCl (Vedeo, St. Joseph, MO) was added to drinking water at a final concentration of 1.1 mg/ml (18).

Cells and Culture Conditions. The C1300 neuroblastoma cell line was established from a spontaneous tumor, which arose in an A/Jax mouse in 1940 (19). The C1300(Muγ) ‡2- line, here referred to as C1300(Muγ), was derived by transfection of C1300 cells with the murine IFN-γ gene using the IFN-γ expression retrovirus pSVX(MuγΔAs) (14). Both lines carry the MHC haplotype *H-2K*^{k}, *I-A*^{k}, *I-E*^{k}, *D*^{d}. The SVEC 4-10 murine endothelial cell line, here referred to as SVEC, was kindly provided by M. Edidin (Department of Biology, The Johns Hopkins University, Baltimore) and was derived by immortalization of lymph node endothelial cells from a C3H (*H-2*^{k}) mouse with simian virus 40 (15). C1300 and SVEC cells were grown in modified Eagle's medium (MEM) sup-

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Abbreviations: dgA, deglycosylated ricin A chain; IFN-γ, interferon γ; MHC, major histocompatibility complex.

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plemented with 10% (vol/vol) fetal calf serum (FCS), 2.4 mM L-glutamine, 200 units of penicillin and 10 μg of streptomycin per ml, 100 μM nonessential amino acids, 1 μM sodium pyruvate, and 18 μM Hepes. C1300(Muγ) cells were maintained in the same medium supplemented with G418 (1 mg/ml) (Geneticin; Sigma). Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10% CO_2 in regular tissue culture flasks or, when large quantities were required for *in vivo* experiments, in cell factories (Baxter, Grand Prairie, TX). For some experiments, SVEC cells were cultured with recombinant IFN-γ, tumor conditioned medium, or neutralizing anti-IFN-γ antibody as described (13).

Monoclonal Antibodies. The M5/114.15.2 (here referred to as M5/114) and 11-4.1 hybridomas were purchased from the American Type Culture Collection and were grown in MEM/10% FCS. The antibodies were purified from culture supernatant by precipitation in 50% ammonium sulfate and affinity chromatography on protein G (M5/114) or protein A (11-4.1). The rat IgG2b antibody, M5/114, detects an epitope on I-A^d and I-E^d molecules, hereafter referred to collectively as Ia^d, on cells from BALB/c mice (20) and also cross-reacts with an epitope on I-E^k molecules on induced SVEC cells (13). The mouse IgG2a antibody 11-4.1 recognizes H-2K^k but not H-2K^d molecules (21) and so binds to H-2K^k on C1300 and C1300(Muγ) cells but is unreactive with MHC antigens from BALB/c mice. A rat IgG2b anti-human CD7 antibody, Campath-2, was generously provided by G. Hale (Department of Pathology, University of Cambridge, Cambridge, England) and was used as the isotype-matched control antibody for M5/114. A mouse IgG2a anti-human CD7 antibody, WT-1, was the kind gift of W. Tax (Sint Radbondzeikenhuis, Nijmegen, The Netherlands) and was used as a control for 11-4.1.

Preparation of Deglycosylated Ricin A (dgA) Chain. The ricin A chain was purified by the method of Fulton *et al.* (22). dgA was prepared as described (17). For conjugation with antibodies, the A chain was reduced with 5 mM dithiothreitol (DTT) and subsequently separated from DTT by gel filtration on a column of Sephadex G-25 in phosphate-buffered saline (pH 7.5) containing 2 mg of Na_2EDTA per ml.

Preparation of Immunotoxins. IgG immunotoxins were prepared by using the 4-succinimidyloxycarbonyl α-methyl(2-pyridyldithio)toluene linking agent described by Thorpe *et al.* (17). Briefly, 4-succinimidyloxycarbonyl α-methyl(2-pyridyldithio)toluene dissolved in dimethylformamide was added to the antibody solution [7.5 mg/ml in borate buffer (pH 9.0)] to give a final concentration of 0.11 mM. After 1 hr, the derivatized protein was separated from unreacted material by gel chromatography on a Sephadex G-25 column and mixed with freshly reduced ricin A chain. The solution was concentrated to ≈3 mg/ml and allowed to react for 3 days. Residual thiol groups were inactivated by treating the immunotoxin with 0.2 mM cysteine for 6 hr. The solution was then filtered through a Sephacryl S-200 HR column in 0.1 M phosphate buffer (pH 7.5) to remove unreacted ricin A, cysteine, and aggregates. Finally, the immunotoxin was separated from free antibody by chromatography on a blue Sepharose CL-6B column equilibrated in 0.1 M sodium phosphate buffer (pH 7.5) according to the method of Knowles and Thorpe (23). All immunotoxin preparations contained >90% 180-kDa product consisting of one molecule of IgG and one molecule of ricin A chain, as assessed by analytical SDS/PAGE.

Cytotoxicity Assays. C1300, C1300(Muγ), and SVEC cells suspended at 10^5 cells per ml in MEM/10% FCS were distributed in 100-μl vol into the wells of flat-bottomed microtiter plates. For some assays, SVEC cells were suspended in C1300- or C1300(Muγ)-conditioned medium or MEM supplemented with recombinant IFN-γ as indicated. Immunotoxins in the same medium were added (100 μl per well) and the plates were incubated for 24 hr at 37°C in an atmosphere of humidified 10% CO_2/90% air. After 24 hr, the cells were pulsed with [^{3}H]leucine (2.5 μCi per well; 1 Ci = 37 GBq) for another 24 hr. The cells were then harvested onto glass fiber filters with a Titertek harvester and the radioactivity on the filters was measured with a liquid scintillation spectrometer (LKB; Rackbeta). The percentage of reduction in [^{3}H]leucine incorporation, as compared with untreated control cultures, was used as the assessment of killing.

Antitumor Experiments. For establishment of solid tumors, a mixture of 1.4×10^7 C1300 cells and 6×10^6 C1300(Muγ) cells in 200 μl of MEM/30% FCS was injected subcutaneously into the right anterior flank of BALB/c *nu/nu* mice. Fourteen days later, when the tumors had grown to 0.8–1.2 cm in diameter, the mice were separated into groups of 5–10 animals and injected intravenously with 200 μl of immunotoxins, antibodies, or diluent. Perpendicular tumor diameters were measured at regular intervals and tumor volumes were estimated according to the following equation (24):

$$\text{volume} = \frac{\text{smaller diameter}^2 \times \text{larger diameter} \times \pi}{6}.$$

For histopathological analyses, animals were killed at various times after treatment and the tumors were excised immediately into 4% (vol/vol) formalin. Paraffin sections were cut and stained with hematoxylin and eosin.

RESULTS

Cytotoxicity of Immunotoxins to Activated Endothelial Cells and Tumor Cells *in Vitro*. The two immunotoxins M5/114-dgA and 11-4.1-dgA were approximately equally potent at inhibiting protein synthesis by their appropriate target cells *in vitro*, as shown in Fig. 1. M5/114-dgA inhibited protein synthesis of SVEC cells activated with recombinant IFN-γ or C1300(Muγ)-conditioned medium by 50% at 0.1–0.3 nM (Fig. 1 *Upper*). Similarly, 11-4.1-dgA inhibited protein synthesis by cells recovered from subcutaneous C1300/C1300(Muγ) tumors by 50% at 0.2 nM (Fig. 1 *Lower*). The cytotoxic effects of both immunotoxins were antigen specific, since isotype-matched immunotoxins prepared from antibodies of irrelevant specificity (human CD7) were not toxic to either cell type at 100 nM. Also, neither immunotoxin was toxic to nontarget cells, including unstimulated endothelial cells, at 100 nM. Importantly, M5/114-dgA, which reacts with I-E^k antigens expressed weakly by C1300(Muγ) cells (13), was not toxic to tumor cells at concentrations below 10 nM (Fig. 1 *Lower*), which strongly suggested that any *in vivo* antitumor effects mediated by this immunotoxin must be via an indirect mechanism, probably disruption of blood supply. Campath-2 and WT-1 immunotoxins were specifically cytotoxic to $CD7^+$ human T-cell lines (unpublished data).

Antitumor Effects in Mice Bearing Large Solid Tumors. A single intravenous injection of the anti-tumor endothelial cell immunotoxin M5/114-dgA into mice bearing large (≥1 cm in diameter) solid tumors induced potent, dose-dependent antitumor effects (Fig. 2). Responses were minor and transient in animals treated with 20 μg of M5/114-dgA but, at a dose of 40 μg, marked regressions were achieved in all animals. Tumors collapsed to an average of a quarter of their initial volume, sometimes reaching almost unmeasurable dimensions, before regrowing 7–10 days later. Higher doses (≥100 μg) of M5/114-dgA were highly effective but were toxic in some cases. By contrast, a high dose (100 μg) of the tumor-specific immunotoxin 11-4.1-dgA induced only minor transient effects on tumor growth, but when the two immunotoxins were used in combination, synergistic antitumor effects were achieved and five of eight of the treated animals

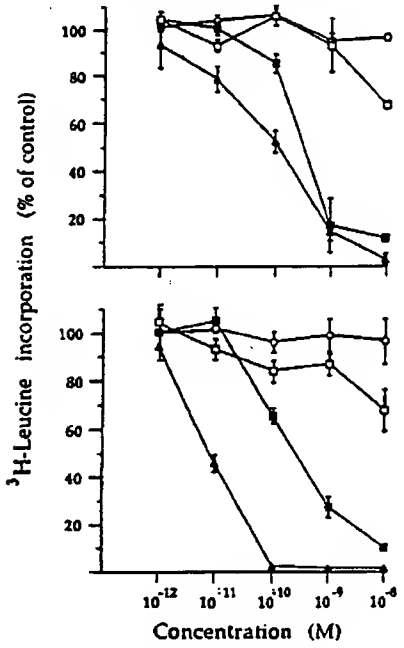

FIG. 1. Cytotoxicity of anti-class II and anti-H-2K^k immunotoxins to target cells *in vitro*. (*Upper*) SVEC cells were incubated for 48 hr with M5/114-dgA after having incubated them for 72 hr in regular medium (○), medium supplemented with recombinant IFN-γ (200 international units/ml) (▲), C1300(Muγ)-conditioned medium (■), or C1300(Muγ)-conditioned medium supplemented with neutralizing anti-IFN-γ antibody (200 neutralizing units/ml) (□). (*Lower*) Cells recovered from subcutaneous C1300/C1300(Muγ) tumors were incubated for 48 hr with ricin (▲), 11-4.1-dgA (■), M5/114-dgA (□), or WT-1-dgA (○). Similar results were obtained in two other experiments. Data points are geometric means of triplicate measurements of [^{3}H]leucine incorporated by the cells during the final 24-hr period of culture expressed as a percentage of the incorporation in untreated cultures. Bars are 1 SEM.

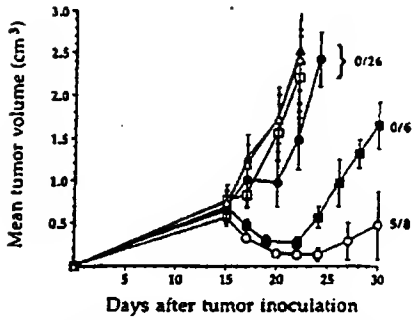

FIG. 2. Induction of tumor regression by anti-class II immunotoxins. C1300(Muγ) tumor-bearing BALB/c *nu/nu* mice were injected intravenously with 20 μg (□) or 40 μg (■) of M5/114-dgA, 100 μg of 11-4.1-dgA (●), or 100 μg of 11-4.1-dgA plus 40 μg of M5/114 dgA (○). Mice in control groups received 200 μl of diluent (△) or 100 μg of an isotype-matched immunotoxin of irrelevant specificity [Campath-2-dgA, anti-human CD7 (▲)]. Mice treated with equivalent doses of unconjugated antibodies (M5/114, 11-4.1) displayed no retardation in tumor growth rate. Immunotoxin doses refer to total protein content. Error bars indicate SEM. Also indicated is number of complete tumor remissions per total number of mice in each experimental group. Similar results were obtained in two other experiments.

cleared their tumors and remained disease-free. These effects were specific since equivalent doses of the unconjugated antibodies or of control immunotoxins of irrelevant specificity had no antitumor effects.

The gross appearance of tumors treated with M5/114-dgA changed dramatically within 2 days (Fig. 3). Massive hemorrhaging of the tumor vascular bed caused the tumors to assume a blackened, bruised appearance reminiscent of the effects when Meth-A fibrosarcomas are treated with bacterial endotoxin (25) or tumor necrosis factor type α (26). Over the next 5–7 days, the tumors collapsed to form a flat scabrous plug that subsequently detached, leaving a small avascular area of scar tissue in animals treated with M5/114-dgA and 11-4.1-dgA. Some tumors became fully coagulated before significant hemorrhaging could occur and so progressed to the final stage illustrated without blackening or scabbing of the tumor mass. In mice given M5/114-dgA alone, the tumors regrew from a ring around the original site and reinvaded the core of dead tumor tissue.

Histological Observations. A study of the time course of the events in tumor-bearing mice treated with M5/114-dgA revealed vascular endothelial cell destruction as the first visible event, occurring as early as 2 hr after administration of the immunotoxin (Fig. 4*a*). Degeneration of the endothelial cell layer induced a wave of platelet adhesion and activation followed by fibrin deposition. By 6 hr, many blood vessels in the tumor were occluded with thrombi and had been stripped of their endothelial cell lining (Fig. 4*b*). At this time, the tumor cells themselves were morphologically unchanged. By 24 hr, all vessels contained mature thrombi and the surrounding tumor cells had pyknotic nuclei (Fig. 4*c*). By 48 hr, massive tumor cell degeneration and autolysis had occurred (Fig. 4*d*).

The cause of tumor regrowth in mice treated with M5/114-dgA alone was investigated by histopathological examination of treated tumors (Fig. 5). Three days after injection of M5/114-dgA, a median section showed hemorrhagic necrosis throughout the tumor, most advanced in the core. However, a thin cuff of tumor, 5–10 cells thick, survived on the extreme periphery (Fig. 5 *Inset*) of the tumor and it was these cells that subsequently proliferated to cause the regressions in animals treated with M5/114-dgA alone.

Lack of Damage to Normal Tissues. Previous studies had shown that class II antigens are expressed by several normal cell populations in BALB/c *nu/nu* mice, including some

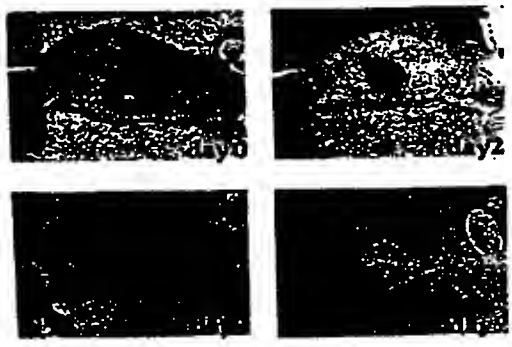

FIG. 3. Gross appearance of subcutaneous tumors treated with anti-class II immunotoxins. Mice were inoculated with tumor cells and treated with 40 μg of M5/114-dgA as described. Before treatment (day 0), tumors were pink/purple, indicating florid vascularization. Two days after treatment, massive intratumoral hemorrhage caused a blackened discoloration, and by day 7 the tumor mass had largely collapsed into a scabrous tissue plug, which later dropped off to reveal a white, avascular nodule of dead tumor tissue (day 9). Representative mice at different stages of therapy are shown.

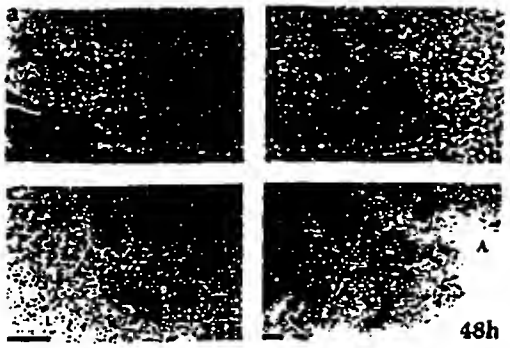

FIG. 4. Time course of vascular thrombosis and tumor necrosis after administration of M5/114-dgA. (*a*) Two hours: a few endothelial cells had become denuded with exposure of the underlying subendothelial extracellular matrix (arrows). Platelet (PT) adhesion and aggregation on the damaged areas was visible. (*b*) Six hours: many vessels had become completely occluded by mature fibrin thrombi (T). (*c*) Twenty-four hours: all vessels were thrombotic and surrounding tumor cells had pyknotic nuclei (P). (*d*) Forty-eight hours: tumor necrosis had advanced and areas of pyknosis, karyolysis (K), and autolysis (A) were apparent. Hematoxylin and eosin stain. (Bars: *a* and *b*, 15 μm; *c* and *d*, 60 μm.)

intestinal and renal tubular epithelial cells, B cells, a minority of bone marrow cells, Kupffer cells, Langerhans cells, and macrophages in most organs (13). However, therapeutic doses (≥40 μg) of M5/114-dgA did not cause detectable damage to class II-positive epithelial cells or to Kupffer or Langerhans cells, as assessed by histopathological analyses at various times after treatment (unpublished data). It would be expected that readily accessible class II-positive cells in primary and secondary lymphoid organs would be killed by the immunotoxin, but these effects were apparently temporary because, 20 days after treatment, all mature bone marrow cell populations and splenic B-cell compartments were normal (unpublished data).

DISCUSSION

The findings from this experimental model validate the concept of tumor vascular targeting and, in addition, demonstrate that this strategy is complementary to that of direct tumor targeting. The theoretical superiority of vascular targeting over the conventional approach was established by comparing the *in vivo* antitumor effects of two immunotoxins—one directed against tumor vascular endothelium and the other directed against the tumor cells themselves—in the same model. The immunotoxins were equally potent against their respective target cells *in vitro* but, while 100 μg of the tumor-specific immunotoxin had practically no effect against large solid C1300(Muγ) tumors, as reported previously in other systems (27, 28), as little as 40 μg of the anti-tumor endothelial cell immunotoxin caused complete occlusion of the tumor vasculature and dramatic tumor regressions.

FIG. 5. Microscopic appearance of tumors from mice treated with anti-class II immunotoxin 72 hr earlier. (Bar = 1.0 mm.) (*Inset*) Almost the entire tumor mass is necrotic; only a thin cuff of tumor cells at the extreme tumor periphery adjacent to normal tissue survives (arrows). Hematoxylin and eosin stain. (Bar = 40 μm.)

Despite causing thrombosis of all blood vessels within the tumor mass, the anti-tumor endothelial cell immunotoxin was not curative because a small population of malignant cells at the tumor–host interface survived and proliferated to cause the observed relapses 7–10 days after treatment. The proximity of these cells to intact capillaries in adjacent skin and muscle suggests that they survived because they could derive their nutrition from the extratumoral blood supply, which was not induced to express class II antigens (13). However, these same tumor cells could be killed by the anti-tumor immunotoxin, probably because they were readily accessible to the immunotoxin because of the florid vasculature and lower interstitial pressure in those regions of the tumor (29). Importantly, the complementary nature of the killing achieved with the antitumor vasculature and anti-tumor immunotoxins resulted in improved antitumor effects when both reagents were administered. Thus, over half of the mice receiving both immunotoxins achieved lasting complete remissions.

The time course study demonstrated that the anti-class II immunotoxin exerted its antitumor activity via the tumor vasculature since endothelial cell detachment and diffuse intravascular thrombosis clearly preceded any changes in tumor cell morphology. In contrast with the anti-tumor immunotoxin, the onset of tumor regression in animals treated with the anti-tumor endothelial cell immunotoxin was rapid. Massive necrosis and tumor shrinkage were apparent 48–72 hr after injection. Focal denudation of the endothelial lining was evident within 2–3 hr, in keeping with the finding that protein synthesis by IFN-induced endothelial cells is reduced by 10% within 2 hr (unpublished data). Because only limited endothelial damage is required to upset the hemostatic balance and initiate irreversible coagulation, many intratumoral vessels became thrombosed quickly thereafter, with the result that tumor necrosis began within 6–8 hr of administration of the immunotoxin. This illustrates several of the strengths of vascular targeting in that an avalanche of tumor cell death swiftly follows destruction of a minority of tumor vascular endothelial cells (9). Thus, in contrast to conventional tumor cell targeting, anti-endothelial immunotoxins could be effective even if they have short serum half-lives and only bind to a subset of tumor endothelial cells. The anti-tumor immunotoxin 11-4.1-dgA killed large numbers of tumor cells, giving rise to discrete islands of necrosis surrounding intact blood vessels (unpublished data). However, many cells in poorly vascularized regions of the tumor were inaccessible to the immunotoxin (13), and this surviving fraction proliferated rapidly so that the overall effect of the anti-tumor immunotoxin on tumor growth was minimal.

MHC class II antigens are not unique to vascular endothelial cells. They are expressed constitutively on B cells, bone marrow myelocytes, cells of monocyte–macrophage lineage, and on some renal and gut epithelial cells in BALB/c *nu/nu* mice (13). It would therefore be anticipated that damage to these normal tissues would result if anti-class II immunotoxin were to be administered. However, anti-class II immunotoxins administered intravenously to antibiotic-treated BALB/c *nu/nu* mice were no more toxic to the mice than are immunotoxins having no reactivity with mouse tissues. There are a number of possible explanations for this

surprising result. First, anti-class II antibodies injected intravenously did not appear to reach the epithelial cells or the monocytes–macrophages in organs other than the liver and spleen (13). Presumably, this is because the vascular endothelium in most organs is tight, not fenestrated as it is in the liver and spleen, and so the antibodies must diffuse across basement membranes to reach the class II-positive cells. Second, hepatic Kupffer cells and probably other cells of monocyte–macrophage lineage were not killed by the anti-class II immunotoxin even though it binds to them (13). No morphological changes in the Kupffer cells were visible even several days after administration of the immunotoxin. Cells of monocyte–macrophage lineage are generally resistant to ricin A-chain immunotoxins (30), probably because internalized immunotoxins are routed directly to lysosomes and metabolized as part of the cells' degradative physiologic function. Third, although B cells and bone marrow myelocytes were probably killed by the immunotoxin, they were efficiently replaced from the stem cell pool because early bone marrow progenitor cells do not express class II antigens (31).

It was important to maintain mice on oral antibiotics to prevent toxicity of anti-class II immunotoxins to the small intestine. In animals not given tetracycline HCl, some ileal villous endothelial cells were induced to express class II antigens by IFN-γ secreted by thymus-independent intraepithelial lymphocytes responding to gut flora. Antibiotic treatment diminished local T-cell accumulation and abolished endothelial cell class II antigen expression (13). Tetracycline itself had no effects on tumor growth because mice injected with diluent or control immunotoxins showed the same kinetics of tumor growth whether or not they were given the antibiotic (unpublished data).

Although this model system cannot be directly transferred to the clinic, the findings described in this report demonstrate the therapeutic potential of the vascular targeting strategy against large solid tumors and highlight the need for antibodies recognizing endothelial cells in human tumors. Numerous differences between tumor blood vessels and normal ones have been documented (9, 29, 32), which suggest that the approach could be applicable in humans. Tumor endothelial markers could potentially be induced directly by tumor-derived angiogenic factors (33) or cytokines (34, 35) or could relate perhaps to the rapid proliferation (36) and migration (33) of endothelial cells during neovascularization. Indeed, several candidate anti-tumor endothelial cell antibodies have recently been described. The antibodies FB-5, against endosialin (11), and E9 (12) have been reported to be highly selective for tumor vascular endothelial cells. Two related antibodies, TEC-4 and TEC-11, raised in this laboratory against carcinoma-stimulated human endothelial cells, show strong reactivity against vascular endothelial cells in a wide range of malignant tumors but little or no staining of vessels in benign tumors or normal tissues (F.J.B., P. Tazzari, P. Amlot, A. F. Gazdar, E. S. Vitetta, and P.E.T., unpublished data). Vascular targeting could therefore become a valuable approach to the therapy of disseminated solid cancers for which there are currently no effective treatments.

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30. We thank L. Luo and Y. N. Jan for the transgenic strains carrying *Drac1* and *Dodc42* derivatives, T. Uemura for the *elav-GAL4* strain, A. Nose for monoclonal antibody 1D4, A. DiAntonio and E. Giniger for the plasmids containing the *elav* promoter and *kinesin* gene, respectively, C.-S. Yoon for technical instruction, and S. Yoshida, A. Chiba, and all members of our department for comments and discussions. Supported by a Grant-in-Aid for Priority Area from the Ministry of Education, Science, Sports and Culture and the research grant for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan.

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Tumor Infarction in Mice by Antibody-Directed Targeting of Tissue Factor to Tumor Vasculature

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Selective occlusion of tumor vasculature was tested as a therapy for solid tumors in a mouse model. The formation of blood clots (thrombosis) within the tumor vessels was initiated by targeting the cell surface domain of human tissue factor, by means of a bispecific antibody, to an experimentally induced marker on tumor vascular endothelial cells. This truncated form of tissue factor (tTF) had limited ability to initiate thrombosis when free in the circulation, but became an effective and selective thrombogen when targeted to tumor endothelial cells. Intravenous administration of the antibody-tTF complex to mice with large neuroblastomas resulted in complete tumor regressions in 38 percent of the mice.

As a strategy for cancer therapy, the use of immunoconjugates that selectively occlude the vasculature of solid tumors offers several theoretical advantages over immunoconjugates that target tumor cells directly. First, because tumor cells depend on a blood supply, local interruption of the tumor vasculature will produce an avalanche of tumor cell death (*1*). Second, the tumor vascular endothelium is in direct contact with the blood, whereas the tumor cells themselves are outside the bloodstream and, for the most part, are poorly accessible to immunoconjugates (*2*). Third, tumor vascular endothelial cells are not transformed and are unlikely to acquire mutations that render them resistant to therapy.

We explored the feasibility of treating solid tumors by targeting human tissue factor (TF) to tumor vascular endothelium in a mouse model. TF is the major initiating receptor for the thrombogenic (blood coagulation) cascades (*3*). Assembly of cell surface TF with factor VII/VIIa generates the functional TF:VIIa complex. This complex rapidly activates the serine protease zymogens factors IX and X by limited proteolysis, leading to the formation of thrombin and, ultimately, a blood clot. A recombinant form of TF has been constructed that contains only the cell surface domain (*4*). This truncated TF (tTF) is a soluble protein with a factor X–activating activity that is about five orders of magnitude less than that of native transmembrane TF in an appropriate phospholipid membrane environment (*5*). This is because the TF:VIIa complex binds and activates factors IX and X far more efficiently when associated with a negatively charged phospholipid surface (*5*, *6*). We reasoned that, by using an antibody to target tTF to tumor vascular endothelium, the tTF would be brought into proximity with a cell surface so as to recover in part its native function and locally initiate thrombosis. Such an antibody-tTF conjugate (or "coaguligand") would selectively thrombose tumor vasculature.

To test this concept, we used a mouse model in which the tumor vascular endothelium expresses a marker that is lacking on the normal vascular endothelium (*7*). Naturally occurring markers of tumor vascular endothelium have not been identified in mice, although some strong candidates have been identified for humans (see below). In our model, C1300(Muγ) mouse neuroblastoma cells that have been stably transfected with the murine interferon-γ (IFN-γ) gene are grown as a solid subcutaneous tumor in BALB/c *nu/nu* mice. The IFN-γ secreted by the tumor cells induces local expression of major histocompatibility complex class II antigens ($I\text{-}A^d$ and $I\text{-}E^d$) on the tumor vascular endothelium. Class II antigens are absent from normal vascular endothelium in mice, although they are present on B lymphocytes, monocytes, and some epithelial cells.

To target tTF to $I\text{-}A^d$ on tumor vascular

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endothelium, we prepared a bispecific antibody with the Fab' arm of the B21-2 antibody, specific for I-A^d, linked to the Fab' arm of the 10H10 antibody, specific for a noninhibitory epitope on the C-module of tTF (8). This bispecific antibody, B21-2/10H10, mediated the binding of tTF in an antigen-specific manner to I-A^d on A20 mouse B-lymphoma cells in vitro (Fig. 1A). When mouse plasma was added to A20 cells to which tTF had been bound by B21-2/10H10, it coagulated rapidly. Fibrin strands were visible 36 s after the addition of plasma to antibody-treated cells, as compared with 164 s when plasma was added to untreated cells (Fig. 1B). This enhanced coagulation was observed only when tTF was bound to the cells; no effect on coagulation time was seen with cells incubated with tTF alone, with homodimeric $F(ab')_2$, with Fab' fragments, or with tTF plus bispecific antibodies that had only one of the two specificities needed for binding tTF to A20 cells.

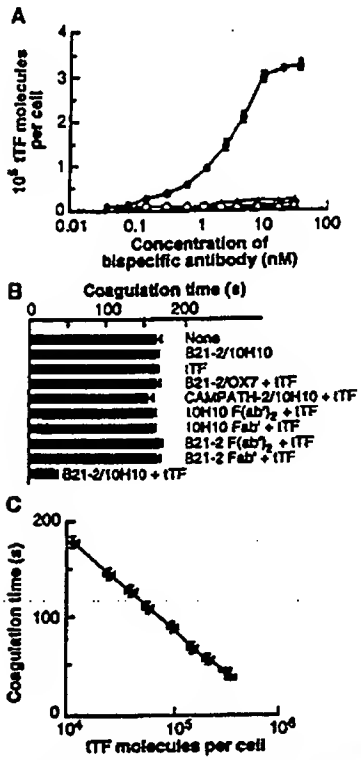


Fig. 1. Effect of the binding of tTF to A20 cells on plasma coagulation. (A) Binding of tTF to A20 cells by means of a bispecific antibody. A20 cells (10^5 cells, 100 μl) were incubated with antibodies B21-2/10H10 (●), CAMPATH-2/10H10 (△), B21-2/OX7 (▲), or phosphate-buffered saline (○) plus an excess of ^{125}I-labeled tTF for 1 hour at 4°C in the presence of 0.2% (w/v) sodium azide and bovine serum albumin (2 mg/ml). The cells were centrifuged through a mixture of phthalate oils (25). The number of bound tTF molecules per cell was calculated from the radioactivity associated with the pellet. (B) Induction of coagulation by cell-bound tTF. A20 cells (10^5 cells, 100 μl) were incubated with antibodies (0.33 μg) and tTF (0.17 μg) for 1 hour at 4°C. Calcium chloride (12.5 mM) and citrated mouse plasma were added to the cells, and the time until the first fibrin strands formed was recorded. (C) Relation between the number of bound tTF molecules and plasma coagulation time. A20 cells (10^5 cells, 100 μl) were incubated with varying concentrations of B21-2/10H10 plus an excess of tTF for 1 hour at 4°C in the presence of sodium azide and were then washed and warmed to 37°C. Calcium chloride (12.5 mM) and citrated mouse plasma (a different batch from that in (B)) were added to the cells, and the time until the first fibrin strands formed was recorded. The number of tTF molecules bound to the cells was determined in a parallel experiment with ^{125}I-labeled tTF. Values (±SD) represent the means of three measurements.

There was a linear relation between the logarithm of the number of tTF molecules bound to the cells and the rate of plasma coagulation by the cells (Fig. 1C). In the presence of cells alone, plasma coagulated in 190 s, whereas at 300,000 molecules of tTF per cell, the coagulation time was 40 s. Even with only 20,000 molecules per cell, coagulation was faster (140 s) than with untreated cells. These in vitro experiments showed that the thrombogenic potency of tTF is enhanced by cell surface proximity mediated through antibody-directed binding to class II antigens on the cell surface.

A histological study was performed to determine whether intravenous administration of the B21-2/10H10-tTF coaguligand induced selective thrombosis of tumor vasculature in mice bearing subcutaneous C1300(Muγ) neuroblastomas 0.8 to 1.0 cm in diameter (9) (Fig. 2). Within 30 min, all vessels throughout the tumor were thrombosed, containing occlusive platelet aggregates, packed erythrocytes, and fibrin. At this time, tumor cells were histologically indistinguishable from tumor cells of untreated mice. After 4 hours, however, there were signs of tumor cell injury. The majority of tumor cells had separated from one another and had pyknotic nuclei, and the tumor interstitium commonly contained erythrocytes. By 24 hours, the tumor showed advanced necrosis, and by 72 hours, the entire central region of the tumor had condensed into amorphous debris. In contrast, there was no visible thrombosis of tumor vessels in mice 30 min after injection with equivalent quantities of tTF alone or tTF in combination with control bispecific antibodies (OX7/10H10, CAMPATH-2/10H10, or B21-2/OX7) that had only one of the two specificities needed for binding of tTF to I-A^d. Similarly, no thromboses were found in nontransfected C1300 tumors, where the endothelium lacks I-A^d.

These experiments indicated that the predominant occlusive effect of the B21-2/10H10-tTF coaguligand on tumor vessels is

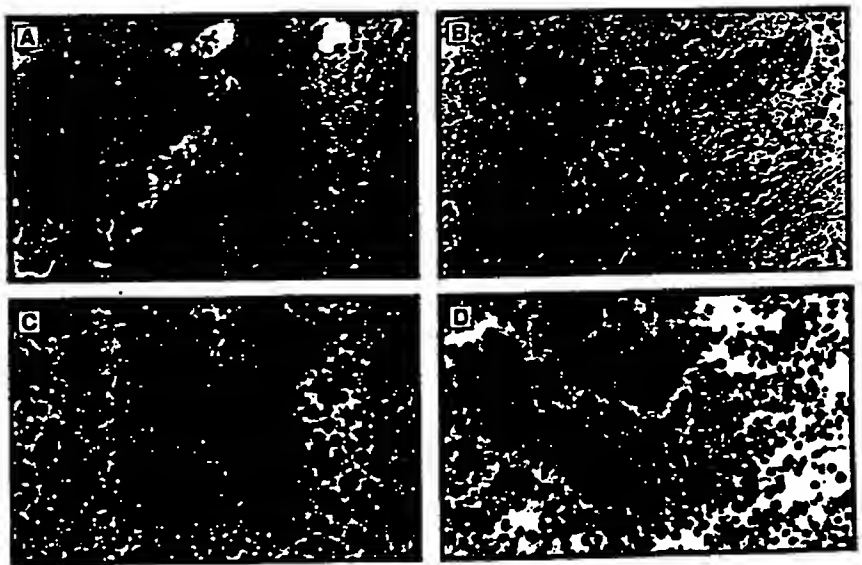


Fig. 2. Histologic analysis of neuroblastomas in mice treated with B21-2/10H10-tTF coaguligand. (A) Before injection (0 hours), the blood vessels are intact and the tumor cells appear normal. (B) At 0.5 hours, the blood vessels throughout the tumor are thrombosed and the tumor cells are normal. (C) At 4 hours, there are dense thrombi in all tumor vessels and the tumor cells are separating from one another, undergoing nuclear pyknosis and cytolysis. Erythrocytes are present in the tumor interstitium. (D) At 24 hours, there is advanced necrosis uniformly throughout the tumor. Arrows indicate blood vessels. Scale bar, 50 μm.

mediated through binding to class II antigens on tumor vascular endothelium. Nevertheless, a nonspecific thrombotic action of tTF was discernible in tumor vessels at later times; in tumors from mice that had been injected 24 hours previously with tTF alone or tTF mixed with the control bispecific antibody OX7/10H10, an average of 40% and 60% of the vessels were thrombosed, respectively. These were most prevalent in the tumor core. It is possible that the resident thrombogenic activity of tumor vasculature (*10*) renders these vessels more susceptible to thrombosis even by untargeted tTF. Alternatively, enhanced procoagulant changes might have been induced by the tumor-derived IFN-γ. Coaguligand treatment was well tolerated (*11*); mice lost no weight and retained a normal appearance and level of activity. Neither thrombi nor histological abnormalities were found in the liver, kidney, lung, intestine, heart, brain, adrenals, pancreas, or spleen from the tumor-bearing mice 30 min or 24 hours after administration of coaguligand or free tTF.

We next investigated whether intravenous administration of the B21-2/10H10-tTF coaguligand could inhibit the growth of large tumors (diameter 0.8 to 1.0 cm) in mice (*9*). The pooled results from three separate experiments (Table 1 and Fig. 3) indicate that 38% (8 of 21) of mice receiving B21-2/10H10-tTF coaguligand had complete tumor regressions lasting 4 months or more. A further 24% (5 of 21) had reductions in tumor volume in excess of 50%. These antitumor effects were significantly greater than for all other treatment groups ($P < 0.05$).

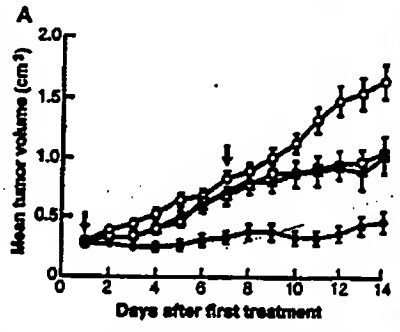
The antitumor effect of the B21-2/10H10-tTF coaguligand was attributable in part to a nontargeted effect of tTF. Tumors in mice receiving tTF alone or mixed with control bispecific antibodies (CAMPATH-2/10H10 or B21-2/OX7) grew significantly more slowly than did tumors in mice receiving antibodies or saline alone ($P < 0.05$). The nontargeted effect of tTF on tumor growth presumably derives from its slight residual thrombogenic activity coupled with the endogenous thrombogenic activity of tumor vessels. However, the nontargeted effect of tTF was weak compared with the coaguligand effect. No mice receiving tTF alone had complete tumor regressions, and only 8% (1 of 12) had a partial remission.

In mice that did not show complete tumor regression after B21-2/10H10-tTF coaguligand treatment, the tumors grew back from a surviving microscopic rim of cells at the periphery of the tumor. Immunohistochemical examination of these tumors revealed that the vascular endothelium at the invading edge of the tumors lacked detectable class II antigens, consistent with a lack of thrombosis of these vessels by the coaguligand, which permitted local tumor cell survival (*12*). Conceivably, coadministration of a drug acting on the tumor cells themselves might improve efficacy, as we observed with another antivascular therapy (*13*, *14*). We previously demonstrated that a powerfully cytotoxic ricin A-chain immunotoxin directed against the tumor cells themselves was virtually devoid of antitumor activity when administered to mice with large C1300(Muy) tumors (*13*, *14*). The lack of activity was a result of the inability of the immunotoxin to gain access to tumor cells in large tumor masses, thus attesting to the comparative effectiveness of coaguligand therapy.

Our experiments illustrate the therapeutic potential of selective initiation of the blood coagulation cascade in tumor vasculature. For clinical application, this strategy will require the identification of target molecules (antigens, receptors) that are present

Table 1. Antitumor effects of B21-2/10H10-tTF coaguligand. The tumor growth index is the ratio of mean tumor volume on day 14 to mean tumor volume on day 0. CR, complete regression; PR, partial remission (>50% decrease in initial tumor volume); NR, no response (<50% decrease in initial tumor volume). Two-tailed *P* values are for differences in tumor volume (day 14) by the Mann-Whitney rank sum test; NS, not significant.

Treatment	*n*	Mean tumor volume (mm^3)		Tumor growth index	Response (%)			*P*	
		Day 0	Day 14		CR	PR	NR	Versus saline	Versus tTF
Saline	27	282	1643	5.8	4	0	96	–	0.05
B21-2/10H10-tTF	21	270	466	1.7	38	24	38	<0.0001	0.0005
tTF	12	285	1054	3.7	0	8	92	0.05	–
B21-2/10H10	13	289	1346	4.7	0	8	92	NS	NS
B21-2/OX7 + tTF	14	293	1027	3.5	7	0	93	0.01	NS
CAMPATH-2/10H10-tTF	8	285	975	3.4	0	0	100	0.002	NS



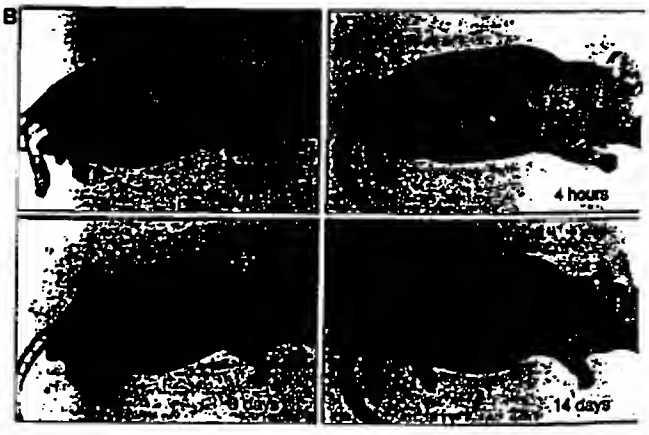


Fig. 3. Tumor regression induced by B21-2/10H10-tTF coaguligand. (A) Mice with C1300-(Muy) tumors (diameter 0.8 to 1.0 cm) were given two intravenous injections of B21-2/10H10-tTF coaguligand spaced 6 days apart (arrows) (●). Mice in control groups received equivalent doses of tTF alone (□), CAMPATH-2/10H10 plus tTF (△), or phosphate-buffered saline (○). Tumor responses in mice that received B21-2/OX7 and tTF were similar to those in mice that received tTF alone and are not shown. Administration of B21-2/10H10 alone did not affect tumor growth. Each group contained 12 to 27 mice (see Table 1 for details of individual groups). Points represent the mean tumor volume per group (±SEM). (B) Gross appearance of subcutaneous tumors after treatment with B21-2/10H10-tTF coaguligand. At the time of treatment (0 hours) the tumor was pink, indicating florid vascularization. After 4 hours, the tumor appeared bruised and blackened. Over the next 8 days the tumor collapsed. By day 14, only fibrous scar tissue was visible in many of the mice.

at sufficient density on the surface of tumor vascular endothelium but absent from normal vascular endothelium (15). Promising candidate molecules for humans include endoglin (16), endosialin (17), an endoglin-like molecule (18), a fibronectin isoform (19), an osteosarcoma-related antigen (20), CD34 (21), collagen type VIII (22), the vascular endothelial cell growth factor (VEGF) receptors (23), and VEGF itself (24). The induction of tumor infarction by targeting a thrombogen to these or other tumor endothelial cell markers represents an intriguing approach to the eradication of primary solid tumors and vascularized metastases.

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8. The B21-2 (TIB-229) hybridoma, secreting a rat immunoglobulin G2b (IgG2b) antibody to the I-A^d antigen, was purchased from the American Type Culture Collection. The CAMPATH-2 antibody is a rat IgG2b antibody to human CD7. The TF9-10H10 antibody (herein referred to as 10H10) is a mouse IgG1 nonneutralizing antibody to human TF [J. H. Morrissey, D. S. Fair, T. S. Edgington, *Thromb. Res.* 52, 247 (1988)]. The MRC OX7 hybridoma (herein referred to as OX7) secretes a mouse IgG1 antibody that recognizes the Thy-1.1 antigen. The bispecific antibodies B21-2/10H10, CAMPATH-2/10H10, OX7/10H10, and B21-2/OX7 were synthesized as described [M. Brennan, P. F. Davison, H. Paulus, *Science* 229, 81 (1985)].
9. To establish solid tumors, we injected 1.5×10^7 C1300(Muy) cells subcutaneously into the right anterior flank of BALB/c *nu/nu* mice (Charles River Labs, Wilmington, MA). When the tumors had grown to ~0.8 cm in diameter, mice were randomly assigned to different experimental groups, each containing four to nine mice. Coagulligands were prepared by mixing bispecific antibodies (150 μg) and tTF (125 μg) in a total volume of 2.5 ml of 0.9% NaCl and incubating at 4°C for 1 hour. Mice received intravenous injections of 0.25 ml of this mixture per 25 g of body weight (that is, 0.6 mg/kg of bispecific antibody plus 0.5 mg/kg of tTF). Other mice received equivalent doses of bispecific antibodies or tTF alone. The injections were performed over ~45 s into one of the tail veins, followed by 200 μl of saline. In the tumor growth-inhibition experiments, the infusions were repeated 6 days later. Perpendicular tumor diameters were measured at regular intervals and tumor volumes were calculated. Differences in tumor volume were tested for statistical significance with the Mann-Whitney rank sum test for two independent samples. For histopathologic analyses, mice were anesthetized with metophane at various intervals after treatment and were exsanguinated by perfusion with heparinized saline. Tumors and normal tissues were excised and immediately fixed in 3% (v/v) formalin. Paraffin sections were cut and stained with hematoxylin and eosin or with Martius Scarlet Blue trichrome for the detection of fibrin. Animal care in all experiments was in accordance with institutional guidelines.
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Geographic Distribution of Endangered Species in the United States

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Geographic distribution data for endangered species in the United States were used to locate "hot spots" of threatened biodiversity. The hot spots for different species groups rarely overlap, except where anthropogenic activities reduce natural habitat in centers of endemism. Conserving endangered plant species maximizes the incidental protection of all other species groups. The presence of endangered birds and herptiles, however, provides a more sensitive indication of overall endangered biodiversity within any region. The amount of land that needs to be managed to protect currently endangered and threatened species in the United States is a relatively small proportion of the land mass.

Previous studies have shown that, on a continental scale, the distributions of well-studied taxa can act as surrogates or indicators for the distribution of poorly studied taxa (*1*–*4*). In contrast, studies of the distribution of "hot spots" of diversity for various taxa within the British Isles suggest that there is very little correlation between the distributions of different taxonomic groups (*5*, *6*). To date, however, no such analysis has been done on a continental or national scale for those species most likely to vanish in the foreseeable future, that is, endangered species. If significant correlations occur in the geographic distributions of different groups of endangered species, it may be possible to use a few well-studied groups as indicators for the purposes of delineating protected areas for other poorly known taxa. The extent to which endangered species are concentrated in hot spots of potential extinctions and the extent to which hot spots for different groups overlap will influence the strategies we adopt to avert species extinctions and the impact of those strategies on other human activities (*7*, *8*). If endangered species are highly concentrated, then fewer areas are likely to experience conflicts between species protection and other activities.

In this study, we used a database of threatened and endangered species in the United States to examine patterns in the geographic distribution of imperiled species (*9*). The database lists the counties of occurrence of all plants and animals protected under the federal Endangered Species Act in the 50 states, plus all species, subspecies, and populations proposed for protection under that statute as of August 1995 (a total of 924 species in 2858 counties). We grouped the species by state, county, and species group (amphibians, arachnids, birds, clams, crustacea, fish, insects, mammals, plants, reptiles, and snails) and then generated dis-

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Advances in Brief

Relationship of Endothelial Cell Proliferation to Tumor Vascularity in Human Breast Cancer

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Abstract

Current studies of tumor angiogenesis rely on the concept that endothelium proliferates 30–40 times faster in tumors than in normal tissues. This evidence is based on histological autoradiographic data largely from animal studies. To assess endothelial cell proliferation in human cancer we used the more sensitive and specific technique of immunohistochemistry. We measured the frequency and distribution of endothelial cell proliferation and examined their relationship to tumor cell proliferation. For the first time, we also correlated endothelial and tumor cell proliferation with tumor vascularity. Twenty breast carcinomas from patients exposed to bromodeoxyuridine 3–8 h prior to surgery were double immunostained using antibodies to CD31 (as a marker of endothelium) and bromodeoxyuridine (as a marker of proliferation). The labeling index (LI) for both tumor and endothelial cells was determined and tumor vascularity was assessed by counting the number of CD31 positive vessels. Endothelial cell proliferation was predominantly at the tumor periphery while tumor cell proliferation occurred throughout the lesion. The mean LIs for endothelium and tumor were 2.2% (range, 0.8–5.3) and 7.3% (range, 1.3–17.1), respectively. There was no correlation between tumor and endothelial cell LI ($P = 0.414$) or between the tumor LI or endothelial cell LI and tumor vascularity ($P = 0.08$ and $P = 0.39$, respectively). These findings suggest that previous studies in animal tumors have significantly overestimated endothelial cell proliferation and that its importance in tumor angiogenesis may be related more to continual remodeling and migration of vessels than to proliferation alone.

Introduction

Angiogenesis is essential for tumor growth and metastasis (1). Endothelial cell matrix remodeling, migration, and proliferation are central to the angiogenic process (2). In marked contrast to the numerous tumor cell kinetic studies there have been few examining endothelial cell proliferations during tumor angiogenesis (3–7). However, based on this small number of reports it has become generally accepted that endothelial cells proliferate 30–40-fold faster in tumor blood vessels than in the vasculature of normal tissue, irrespective of tumor type, growth rate, or size.

However, this oft quoted figure was obtained from endothelial labeling indices derived from histological autoradiographs of animal tissues exposured to tritiated thymidine. To the best of our knowledge in humans only gliomas have been examined in detail (8). The identification of proliferating endothelium by tinctorial stains used in all of these studies permitted recognition only of larger caliber capillaries (a small proportion of the tumor vessels). This difficulty is likely to make the reported endothelial labeling indices inaccurate and also obscures patterns of endothelial cell proliferation and its relationship with the tumor vascularity.

The weakness of previous studies and the paucity of human data warrant reevaluation of this question with more precise techniques. Using immunohistochemistry and antibodies to endothelium and BrdUrd[1] we analyzed endothelial cell proliferation in a series of human breast adenocarcinomas. We report on the frequency and pattern of endothelial cell proliferation and their relationship with tumor cell proliferation and, for the first time, tumor vascularity.

Materials and Methods

Tissue Specimens. Twenty invasive breast carcinomas resected between 1989 and 1991 were taken from the archival files of the Glasgow Royal Infirmary, Scotland. The tumors were derived from patients who had been given i.v. injections of 200 mg bromodeoxyuridine 3–8 h prior to mastectomy. Patients' ages ranges from 40 to 77 years (mean, 61.2 years); 17 were ductal carcinomas of no special type, 1 was a lobular carcinoma, 1 was a medullary carcinoma and 1 was an atypical medullary carcinoma. Of the ductal carcinomas 2 were grade I, 7 were grade II, and 8 were grade III (Nottingham modification of system of Bloom and Richardson) (9). Tumors ranged in diameter from 12 to 120 mm. Nine had histologically confirmed lymph node involvement by tumor.

Immunohistochemistry. Four-μm sections were cut onto silane coated slides and dewaxed. Double immunostaining was performed on sections using streptavidin-biotin-peroxidase and alkaline phosphatase anti-alkaline phosphatase techniques and the antibodies JC70 (Dako) (10) and Bu20a (Dako) (11), respectively (12). For optimal double staining it was necessary to pretreat sections with 12.5 mg protease type XXIV (Sigma)/100 ml phosphate buffered saline for 20 min at 37°C for JC70 and 2 N HCl for 5 min at 60°C for Bu20a. After immunostaining a light hematoxylin counterstain was applied before mounting in aqueous medium. In 10 cases multiple tissue sections were stained. Single BrdUrd immunohistochemistry was performed in parallel sections for all cases in another laboratory.

Morphometry. Labeling indices for both endothelial and tumor cells were determined by scanning the entire tumor section at ×400. In 14 of 20 cases the entire cross-section of the tumor was examined and in 5 of 20, due to their size, at least one-half of the tumor diameter was studied. In one tumor, due to its size of 120 mm, a representative section had to be selected which included the invading tumor margin. A positive endothelial cell was identified on the basis of JC70 positive cytoplasm and/or cell membrane and Bu20a positive nucleus. An endothelial cell was considered negative when an immunonegative nucleus was surrounded by JC70 positive cytoplasm and/or cell membrane. Occasional JC70 immunopositive macrophages and plasma cells were excluded on morphological grounds. The LI of tumor cells was scored by selecting the maximally immunostained area. An average of 1860 endothelial cells and 1398 tumor cells (ranges, 689–2728 and 543–2742, respectively) were counted for each tumor. The vascularity of the tumors was assessed by averaging the number of JC70 positive vessels per mm^2 in the three most vascular areas (13).

Statistical Analysis. Since the data skew and hence cannot be considered normal, a nonparametric Kendall's rank correlation was performed which makes no such assumptions, to assess the relationship between the variables.

Results

Endothelial cell BrdUrd labeling was almost restricted to the infiltrating margin of the tumor with only occasional endothelial cell double immunoreactivity of microvessels within the tumor body. Microvessels were often seen in the body of the tumor without an accompanying endothelial cell nucleus. Proliferating endothelium was

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[1] The abbreviations used are: BrdUrd, bromodeoxyuridine; LI, labeling index.

observed in all types of vessel (*i.e.*, arteriole, venule, narrow and dilated capillaries) (Fig. 1, *A* and *B*). Labeled tumor BrdUrd nuclei were easily distinguishable from labeled endothelial cell BrdUrd nuclei (Fig. 1*C*). Tumor cell labeling showed no similar restricted pattern of proliferation with labeling observed throughout the tumors. The endothelial cell LI ranged from 0.8 to 5.3% (mean, 2.2%) and tumor LI ranged from 1.3 to 17.1% (mean, 7.3%). This tumor cell LI did not differ from the tumor LI established by single BrdUrd immunostaining in another laboratory [correlation score, 138 (n = 20); $P < 0.0001$ (data not shown)]. There was no correlation between endothelial cell and tumor cell LIs ($P = 0.414$) (Fig. 2) or between the endothelial cell LI and tumor vascularity ($P = 0.39$) (Fig. 3). There was no association between tumor cell LI and tumor vascularity ($P = 0.08$) (Fig. 4).

Discussion

In accord with prior studies we observed no relationship between the endothelial cell LI, tumor cell LI, or vascularity (6, 7). However, our findings of a mean endothelial cell LI of 2.2% is in contrast to those reported previously (9%) (3–8). This large discrepancy can be partially explained by the type of vessels examined. Strict morphological definitions based on classical histochemical staining were used to identify vascular endothelium in these previous studies. This necessarily identified endothelial cells only in the larger caliber vessels and therefore the small capillaries, making up most of the tumor vascular network, were excluded. At the time this limitation was recognized but it was assumed that these small budding capillaries, which could not be morphologically identified, would be the vessels likely to proliferate and that the true endothelial cell LI would be still higher (6).

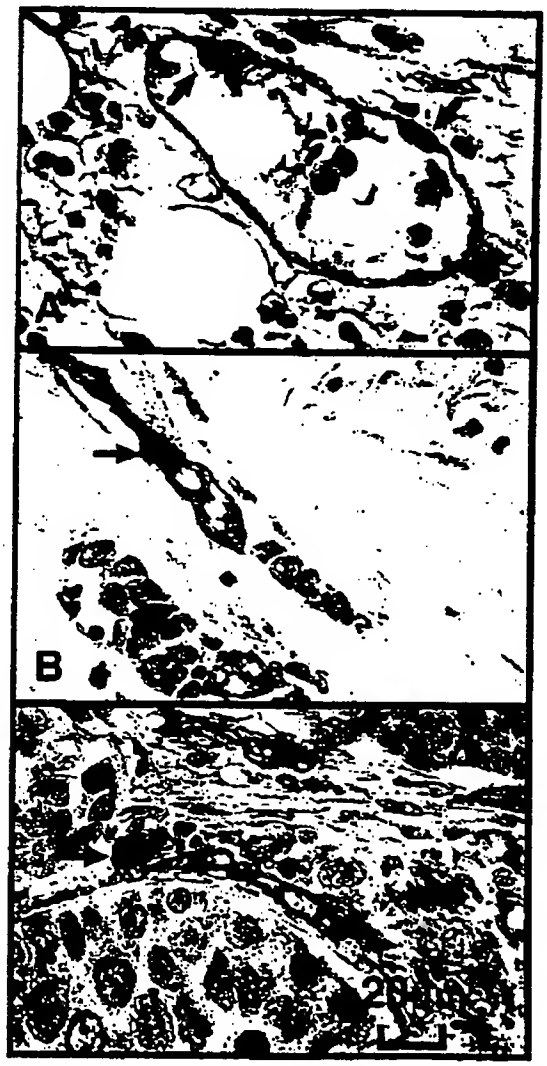

Fig. 1. *A*, dilated positively stained CD31 capillary containing erythrocytes with two labeled BrdUrd endothelial cell nuclei (*arrows*) admixed with neoplastic and inflammatory cells. *B*, CD31 positive microvessel with one labeled BrdUrd endothelial cell nucleus (*arrow*) adjacent to the tumor with negative nuclei. *C*, tumor nests with an immunopositive tumor cell nucleus (*arrow*) and adjacent CD31 positive microvessels with immunonegative nuclei. CD31-diaminobenzidine-immunoperoxidase; BrdUrd-alkaline phosphatase anti-alkaline phosphatase.

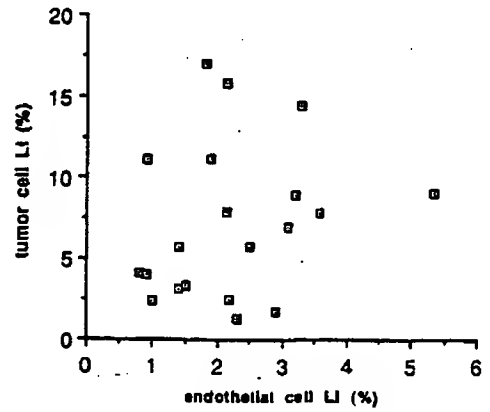

Fig. 2. Tumor cell LI plotted as a function of endothelial cell LI. Score = 25; $P = 0.414$.

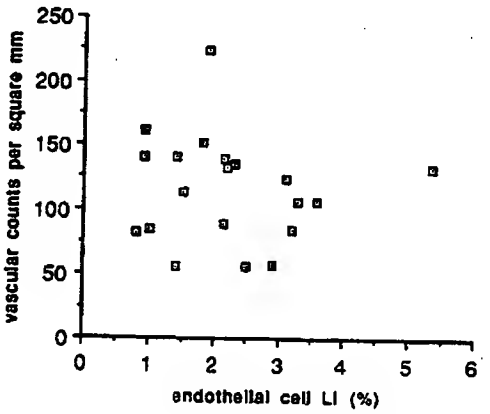

Fig. 3. Vascular counts plotted as a function of endothelial cell LI. Score = 0.084; $P = 0.39$.

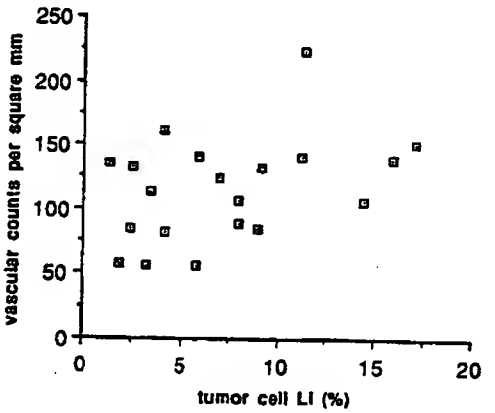

Fig. 4. Vascular counts plotted as a function of tumor cell LI. Score = 53; $P = 0.08$.

In this study immunohistochemistry with an antibody to CD31, the most sensitive vascular endothelial marker to date (14, 15), was used to highlight endothelium. We selected this endothelial marker rather than an antibody to Factor VIII related antigen which is at least as specific but not as sensitive since we wished to identify the complete tumor vasculature. We chose BrdUrd as a marker of proliferation since we could examine large sections of formalin fixed paraffin embedded tumor in contrast to other markers which rely on frozen material. Reliable BrdUrd immunostaining confirmed by comparing double with single BrdUrd immunostaining performed in a different laboratory. We observed that most endothelial cell proliferation occurred at the tumor periphery and that microvessels in the body of the tumor do not proliferate. This pattern of endothelial cell proliferation has been described in the rabbit brain tumor model but not in other animal or human tumors (16) and is not unexpected since endothelial cell proliferation during angiogenesis occurs adjacent to the "parent vessel" and not at the tips of growing capillary sprouts (17). Indeed, this study might have overestimated the true endothelial cell LI since it was common to observe immunoreactive capillaries within the tumor without being able to identify the accompanying endothelial cell nucleus. Because these are liable to be noncycling endothelial cells, inclusion in the LI denominator might further have reduced the endothelial cell LI. One criticism of these findings might be that BrdUrd did not penetrate into the center of the tumor. This possibility is eliminated by the fact that tumor cells were labeled as intensely in the center of the tumor as at its periphery. The BrdUrd in these labeled tumor cells must have passed through the tumor vasculature, the endothelium of which must have necessarily had a prior exposure to BrdUrd.

One of the other major limitations of other studies has been their reliance on animal material. Marked differences between spontaneously arising human and transplanted animal tumors are recognized. Moreover, most of the experimental animal tumors studied were small and fast growing (3–8) with rapid tumor volume doubling times as compared to the human tumors examined in this series. It has been demonstrated that increasing tumor size retards tumor proliferation (18). Furthermore, in several previous studies (7, 8, 19) continuous labeling was performed to optimize LIs, in contrast to the flash labeling carried out in this study. We were able to find limited data on endothelial cell proliferation in nine assorted human tumors (7) and a series of gliomas (8); endothelial LIs ranged from 2 to 37%. However, as far as can be ascertained from published work, only two tumor types had labeling indices exceeding 5%; gliomas which are known for their prominent vascular pattern and a parotid tumor, the precise type not specified. The remaining tumors were assorted lymphomas and carcinomas (the precise histological types appear not to have been published) which had labeling indices from 2.8 to 4.6% within the range but generally higher than those observed in this study.

It is possible that endothelial cell proliferation might be focal or vary diurnally. Furthermore, it is also possible that since we are detecting cells only in S phase, we are missing a substantial number of cycling endothelial cells. However, we have now examined multiple cross-sections of tumors from 20 cases and any significant variation in location or time of endothelial cell proliferation should have emerged.

These findings show that endothelial cell proliferation occurs at the tumor periphery, which suggests that although endothelial cell proliferation must be required for angiogenesis, after endothelial cell division, remodeling and migration of the existing tissue vascular supply establish the functional vasculature within the body of the tumor. Moreover, the association between tumor cell and endothelial cell proliferation only at the invading edge also suggests that growth factors controlling tumor growth are not the same as those involved in endothelial cell growth. This is further supported by the lack of correlation between tumor and endothelial cell LIs. We also observed no correlation between tumor cell LI and tumor vascularity ($P = 0.08$). This is supported by other findings using a similar method of assessing microvessel density and Ki-67 and S-phase fractions as indices of tumor proliferation (20).

This study also challenges the proposal of attacking proliferating tumor endothelium as a unique and universal strategy of tumor treatment on the basis of differential endothelial cell proliferation rates in tumors and normal tissues (21). To target proliferating endothelium much more information will be needed about patterns of proliferation and its importance for tumor angiogenesis. Nevertheless, even a 2- or 3-fold proliferation difference (7, 22) between normal and tumor endothelium might still permit effective targeting of cytotoxic treatment to tumors since although many drugs do not adequately penetrate tumors, drug delivery to the tumor periphery would not compromise cytotoxic efficacy.

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BRIEF COMMUNICATION

Intussusceptive Microvascular Growth in a Human Colon Adenocarcinoma Xenograft: A Novel Mechanism of Tumor Angiogenesis[1]

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Intussusceptive microvascular growth refers to vascular network formation by insertion of interstitial tissue columns, called tissue pillars or posts, into the vascular lumen and subsequent growth of these columns, resulting in partitioning of the vessel lumen. While intussusception has been reported in normal developing organs, its existence in solid tumors has not been previously documented. By observing the growth of the human colon adenocarcinoma (LS174T) *in vivo* for a period of 6 weeks, we demonstrate that intussusception is an important mechanism of tumor angiogenesis. At the leading edge of the tumor, vascular growth was found to occur by both intussusception and endothelial sprouting. In the stabilized regions, intussusception led to network remodeling and occlusion of vascular segments. The formation of some tissue pillars appears to depend on intravascular blood-flow patterns or changes in intravascular shear stress. The rapid vascular remodeling by intussusception could possibly contribute to intermittent blood flow in tumors.

INTRODUCTION

Tumor growth and metastasis are angiogenesis dependent [1]. The commonly accepted mode of tumor angiogenesis is endothelial sprouting. According to this hypothesis neovascularization results from degradation of the basement membrane of the parent vessel, migration and proliferation of endothelial cells, lumen formation, development of sprouts and loops, generation of new basement membrane, and recruitment of pericytes [2–4]. Using intravital microscopy, we have observed an additional mode of tumor angiogenesis: intussusceptive microvascular growth (IMG).[2] IMG is based

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[2] Abbreviations used: IMG, intussusceptive microvascular growth; SCID, severe combined immunodeficient; CAM, chorioallantoic membrane; tPA, tissue plasminogen activator; TGF-β1, transforming growth factor β1; ITS, intervascular tissue structure (one of the extravascular tissue spaces between the vessels of a vascular network); tissue pillar or post, small ITS (diameter <2.5 μm); tissue fold, large vertically running protrusion of the interstitial space projecting with its free tip inside the vascular lumen, or small finger-like projection into the lumen; tissue island, very large ITS (diameter >15 μm); intervascular wall, horizontal connection of two opposite ITSs through a vessel lumen, or slender elongated tissue island located between two vessels.

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on the hypothesis that the capillary network expands by new formation of small intervascular tissue structures (ITSs) called tissue pillars or posts (diameter <2.5 μm) inside the blood vessels that subsequently grow. These pillars span the vascular lumen between opposing walls and thus divide the vessel into two segments (Fig. 1). IMG was first described in the lungs of rabbits [5] and rats [6, 7] and later in a variety of other rat organs [8]. It has also been observed in the chicken chorioallantoic membrane (CAM) microcirculation [9, 10]. Several different modes of IMG were demonstrated in the CAM by *in vivo* video microscopy and analysis of light and electron microscopic serial sections [10]. Recently, intussusceptive growth has been reported during angiogenesis in the mesenteric and peritoneal tissue induced by tumor ascites fluid [11]. Whether growth by intussusception also occurs in solid tumors has not yet been proven. A prerequisite to investigate this question is the availability of an *in vivo* model that permits continuous monitoring of vascular growth in solid tumors. By observing the growth of a human colon adenocarcinoma (LS174T) in the dorsal skinfold chamber in SCID mice [12] at different stages we demonstrate here that IMG is an important mechanism of tumor angiogenesis.

MATERIALS AND METHODS

Tumors and mice. Human tumor xenograft LS174T [human colon adenocarcinoma (ATCC CL 188, American Type Culture Collection, Rockville, MD)] was grown in the dorsal window of 40 SCID mice (bred in the Edwin L. Steele Laboratories animal colony at the Massachusetts General Hospital, Boston, MA). Dorsal skinfold chambers were prepared as described by Leunig *et al.* [12]. In brief, male SCID mice (25–30 g body wt) were anesthetized with 7.5 mg ketamine hydrochloride (Ketalar; Parke-Davis, Morris Plains, NJ) and 2.5 mg xylazine hydrochloride (Anased; Lloyd Laboratories, Shenandoah, IA) per 100 g body wt (sc). The entire back of the animal was shaved and depilated. Two symmetrical titanium frames (weight 3.2 g) were implanted under aseptic conditions to sandwich the extended double layer of skin. One layer of skin was removed in a circular area of approximately 15-mm diameter, and the remaining one was covered with a coverslip and incorporated into one of the frames. Following implantation of the access chamber, animals were allowed to recover for 1–3 days from surgery and anesthesia. Preparations fulfilling the criterion of an intact microcirculation were utilized as sites for tumor cell implantation.

Tumor implantation. For implantation of human colon adenocarcinoma LS174T cells the coverslip of the chamber was removed and 2 μl of a dense tumor cell suspension from the cell culture (2×10^5 cells, viability 95–97%, passage 121–128) was inoculated onto the upper tissue layer of the chamber preparation (striated skin muscle as assessed by histology) and the access chamber was closed again.

In vivo video microscopy. The *in vivo* studies of tumor angiogenesis were performed in mice anesthetized with a mixture of ketamine hydrochloride and xylazine hydrochloride (10:1, 1 ml per 100 g body wt, sc) to prevent animal movement and studies were thus limited to 4 hr. This period was found to be adequate based on previous *in vivo* studies in the chicken CAM where sudden changes could be detected within periods of only a few hours [10]. The tumors were investigated on various days between Days 7 and 40 after tumor cell inoculation. The intravital microscope (Olympus BWH2; Lake Success, NJ) was fitted with a 50×, 0.55 NA objective and a heating pad (Model CN 900 A; Omega, Stamford, CT) to maintain the body temperature of the animal at

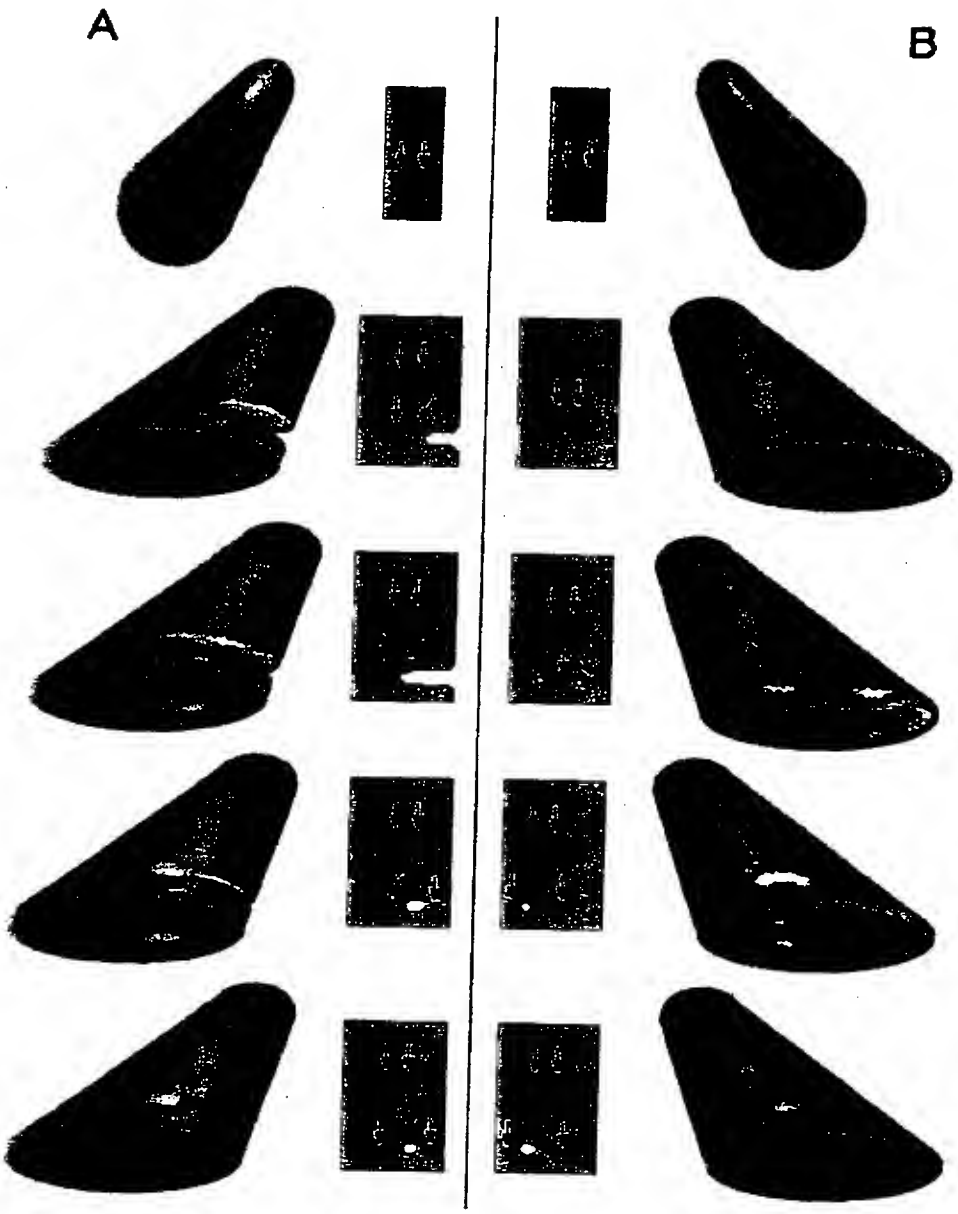


FIG. 1. Modes of tissue pillar formation. 3-D view and top view. Arrows indicate flow. (A) Pillar formation by folding of the capillary wall. A vertical tissue fold is formed projecting inside the vessel lumen. In later stages this fold is thinning due to retraction of endothelial cells. At its central parts the fold grows extremely thin and is finally composed of one endothelial cell extension. Cell membrane fusion within this thin part leads to formation of a transcellular hole that separates the tip of the fold. Further increase of the hole results in formation of a free intraluminal tissue pillar that divides the lumen into two segments. It constitutes a stable column allowing for further dilatation and expansion of the vessel (according to [10]). (B) Pillar formation by connection of two opposing tissue folds projecting intraluminally or due to vascular collapse. After vessel dilatation the two opposing vascular walls come into close contact within the center of the lumen. This can be achieved either by vascular collapse or by formation of two tissue folds projecting intraluminally. In the case of vascular collapse, the region of contact might open up again (right) or become a stable pillar (left). The latter implies formation of interendothelial junctions followed by cell membrane fusion between opposing endothelial leaflets. This results in formation of two transcellular holes allowing for invasion of interstitial tissue and stabilization of the structure (according to [7]). Within regions of intravascular flow divergence, two finger-like tissue folds frequently form projecting inside the lumen from opposite vessel walls. After connection of these folds and cell membrane fusion within the opposing endothelial cells, the interstitial spaces of both folds are connected to form a stable pillar (see Figs. 3 and 4).

37°. Observations of tissue architecture and flow conditions were continuously monitored for periods of up to 4 hr using an intensified CCD camera (Model 2400; Hamamatsu Corp., Middlesex, NJ) and a video recorder (Panasonic AG-6500; Secaucus, NJ). At the end of the study the animals were sacrificed by high-dose anesthesia (sodium pentobarbital, 300 mg/kg ip).

Image analysis. The videotapes were analyzed for changes in morphology and flow patterns in the tumor microcirculation. Still videoframes were digitized at various time points. Image processing was performed using a Macintosh Quadra 650 and the NIH Image 1.54 software (available via FTP at zippy.nimh.nih.gov). Images were subjected to contrast enhancement as well as gradient and smoothing convolutions to emphasize relevant features. The boundaries of blood vessels were found by producing a binary version of each grayscale image; the accuracy of the segmentation was verified by viewing the original tapes and checking vessel morphology and flow direction. The binary images were then processed with an edge detection filter to demarcate the flow boundaries. The binary image was combined with the original grayscale image to produce the final image.

Quantitation of the observed structural alterations. The structural changes detected are described in detail under Results and Discussion and are categorized as follows: sprout formation, pillar formation by folding of the lateral vascular wall, formation of a flow pillar, pillar formation by splitting of large ITSs, fusion of ITSs or pillars, and connection of intraluminal tissue folds with the opposite vascular wall. The six stages are divided as follows: first stage, Weeks 1 (Days 1–7) and 2 (Days 8–14); second stage, Week 3 (Days 15–21); third stage, Week 4 (Days 22–28); fourth stage, Weeks 4 and 5 (Days 28–33); fifth stage, Weeks 5 and 6 (Days 34–37); and sixth stage, Week 6 (Days 38–40). Total observation time (summed over all tumors) of each stage is as follows: stage 1, 8.0 hr (10 tumors); stage 2, 9.5 hr (8 tumors); stage 3, 10.0 hr (8 tumors); stage 4, 6.5 hr (4 tumors); stage 5, 8.75 hr (5 tumors); and stage 6, 7.5 hr (5 tumors). The observation time was measured according to the indication of the video recorder. The structural events are quantified as the number of a certain structural change (e.g., flow pillar formation) observed in one period (e.g., second stage = Week 3) divided by the total observation time of the period (e.g., stage 2 = 9.5 hr): pillars/hr. After studying the general morphology of the tumor at low magnification, it was in most cases only possible to investigate one area per tumor at high magnification (50×), as the maximum observation time per animal was approximately 4 hr due to the limitations of the subcutaneous anesthesia. The areas (size 136 × 140 μm) were selected randomly. In a few animals we were not able to observe any change. Some tumors presented an image of poor quality resulting in a very short observation time.

RESULTS

The first week after tumor cell inoculation was characterized by a rapid expansion of the vascular space into the extravascular one. An overall vasodilatation could be observed in both the host vasculature of the subcutaneous adipose layer covering the striated skin muscle around the tumor and the vessels located on the tumor surface. As a result of this dilatation, extremely wide vessels developed. They were often flat and appeared like "blood lakes" (Fig. 2). Sprouting and pillar formation from the lateral vessel wall quickly divided larger islands of nonvascularized tissue into smaller

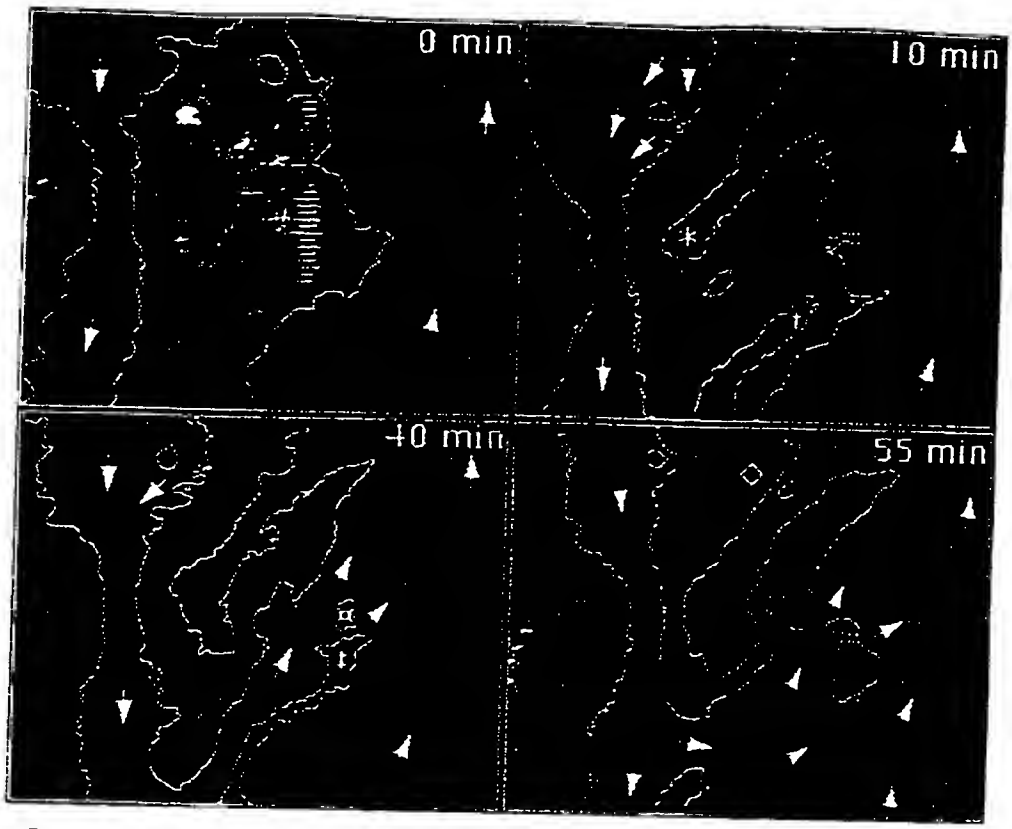
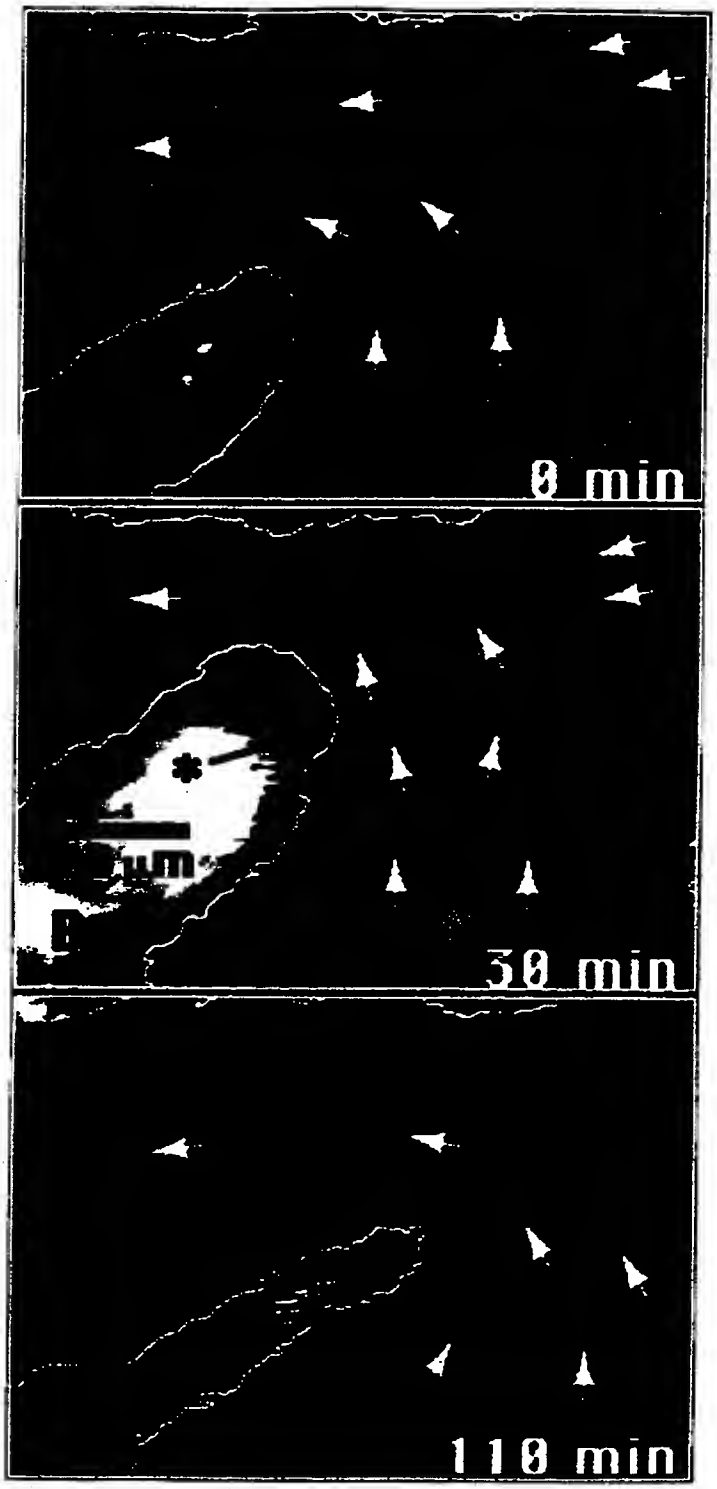


FIG. 2. A typical sequence of events monitored on Day 7. (A) A large island of nonvascularized tissue located between a vessel on the left (average diameter 15 μm) and a blood lake on the right. Flow patterns are indicated by white arrows. (B) After 10 min, two sprouts appear at the center, budding from the upper right (*) and lower left (†) corner of the blood lake. At the same time the tip of a very small fold that was projecting intraluminally at the upper left margin of the island in A has now separated to form a free intraluminal tissue pillar and the left vessel has expanded around it. Both sprouts become more prominent, but they still end blindly and are not yet regularly perfused. In C the process of expansion of the vascular area inside the tissue island has continued. The sprout projecting from below (†) has now elongated and connected to the blood lake at the right; this vessel is now perfused. As the sprout (†) connects with the blood lake, the tissue that previously formed its lower boundary now separates and forms an island in the blood lake (‡). As a second step a small ITS (¤) separates from the intraluminal tip of the right portion of the tissue island that had formed following the sprout connection to the blood lake. In D the process continues with regression of the projection at the lower boundary of sprout (†). Blood now flows freely from left to right across the lower portion. At the same time, a new pillar forms from a small fold that was projecting into the lumen of the right upper sprout near its origin from the blood lake (◇).

FIG. 3. An enormously dilated vessel within the fat tissue near the tumor on Day 14. The convergence of flow from the lower and upper right portions is shown with arrows. (A) A large tissue island forms an acute angle through which the flow must pass. (B) After about 30 min a small fold projecting from below into the vessel lumen (*) can be detected. It forms within the area at the region of convergence, but is not connected to the opposite vessel wall and therefore does not yet form a pillar. It is characterized by a decrease in flow volume at its highest point and an increase in flow around its circumference. White blood cells pass very slowly around the fold and occasionally attach to it transiently. C demonstrates the same fold 2.5 hr later. The flow pattern has changed and blood does not enter from the upper right branch anymore. At this stage, the fold has disappeared, presumably due to the change in local flow conditions. The large tissue fold projecting into the vessel lumen at an acute angle has become more slender and the blood moves around its tip more slowly now.


0 min
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110 min

parts (Fig. 2). Since sprout formation and elongation occurred very quickly we believe that this was not only due to budding from the margins of the tissue island depicted in Fig. 2 but also from expansion of deeper vessels located underneath this island.

The pattern observed during the second week was similar to the first. Sprouting and pillar formation by folding of the vascular wall constituted the dominant structural change in addition to vasodilatation (0.2 sprouts/hr and 0.8 pillars/hr, formed by folding of the vascular wall). However, sporadic signs of vascular remodeling could be detected at the end of the second week. Remodeling refers to rearrangement of the number and position of vascular segments. These rearrangements are accomplished by splitting of existing segments due to insertion of pillars at places of disturbed flow or formation of new segments by division of ITSs. Remodeling is also characterized by a decrease in the number of segments caused by growth and fusion of all types of ITSs. On Day 14, a small fold formed within a region of flow divergence. After changes of the flow pattern, it was, however, removed and subsequently could not form a stable pillar (Fig. 3). Thus starting with the third week, the flow-induced pillar, either being a reversible or stable process, was additionally detected (1.0 flow pillars/hr).

A few days later, at the end of Week 3, pillars frequently formed by splitting of large ITSs (1.7 split pillars/hr). The third week was thus dominated by network remodeling, although, in places, the pattern seen during the first and second weeks was retained. At many sites, vascular diameters decreased, while at others they remained dilated. The formation of a stable pillar within a region of flow divergence could now be demonstrated within a period of 2 hr (Figs. 4A and 4B).

Figure 4 also depicts an intervascular wall that split into a sequence of three ITSs. One of them further divided into two pillars after a few minutes (Fig. 4c). Other signs of remodeling are seen in the middle of the figure, where several vascular segments seem to disappear as they are no longer perfused.

During Week 4 sprouting reached its maximum (3.1/hr). Pillars derived from splitting of ITSs were also frequently detected (2.3/hr), while those originating from folding of the capillary wall were observed with the same frequency as in the first 3 weeks (0.8/hr). Taken together the results demonstrate a growth maximum in the fourth week. However, sprouts formed in this phase seemed to generate a smaller area of vascular space compared to those formed during the first 2 weeks.

At the end of Week 4 to the beginning of Week 5 splitting was detected together with pillar formation by lateral folding of the vascular wall (pillars formed by splitting, 0.9/hr; and pillars formed by folding, 0.8/hr). At the end of Week 5, the network exhibited even stronger signs of vascular remodeling either resulting in formation of

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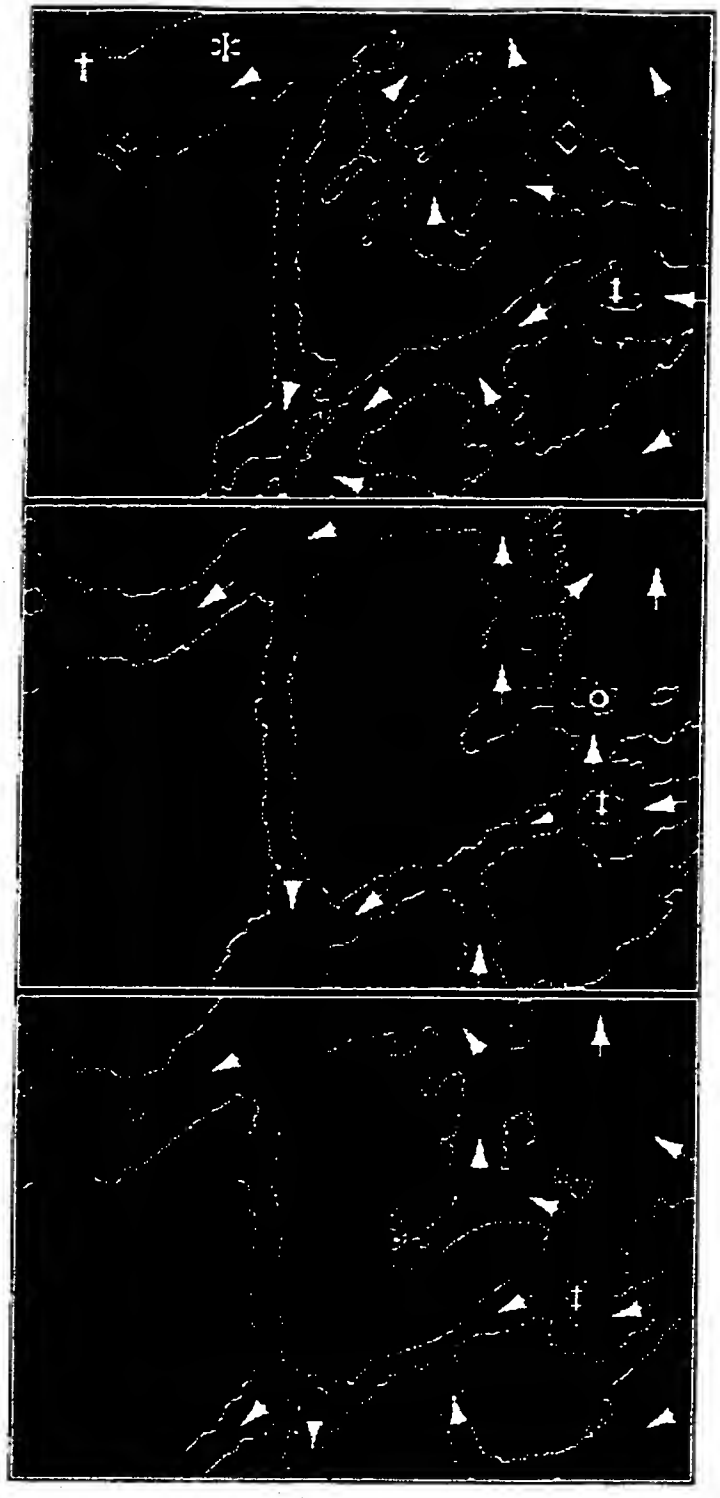
FIG. 4. Vascular remodeling on Day 21. (A) A blood vessel (average diameter 24 μm) enters a branching point on the left on Day 21 (*). Flow is directed in this vessel from right to left and diverges at the branching point following the upper left or lower left vessel branch. At the point of flow divergence a small ITS is already present, further dividing flow into two streams (†). B shows the same tumor vessel about 2 hr later. Within the area of flow divergence a second, very tiny ITS in size range of a tissue pillar has been formed. At the same time several vascular segments in the middle parts of the image seem to be closed as they are not perfused anymore (the mesh marked ‡ serves as a convenient reference point in this sequence since it does not move with respect to the frame). A larger intervascular wall separating two vessels at the right in A (◇) has split into a sequence of at least three ITSs. After a while, one of them (□) has further divided into two tissue pillars allowing fusion of these vessels at an additional site in C.



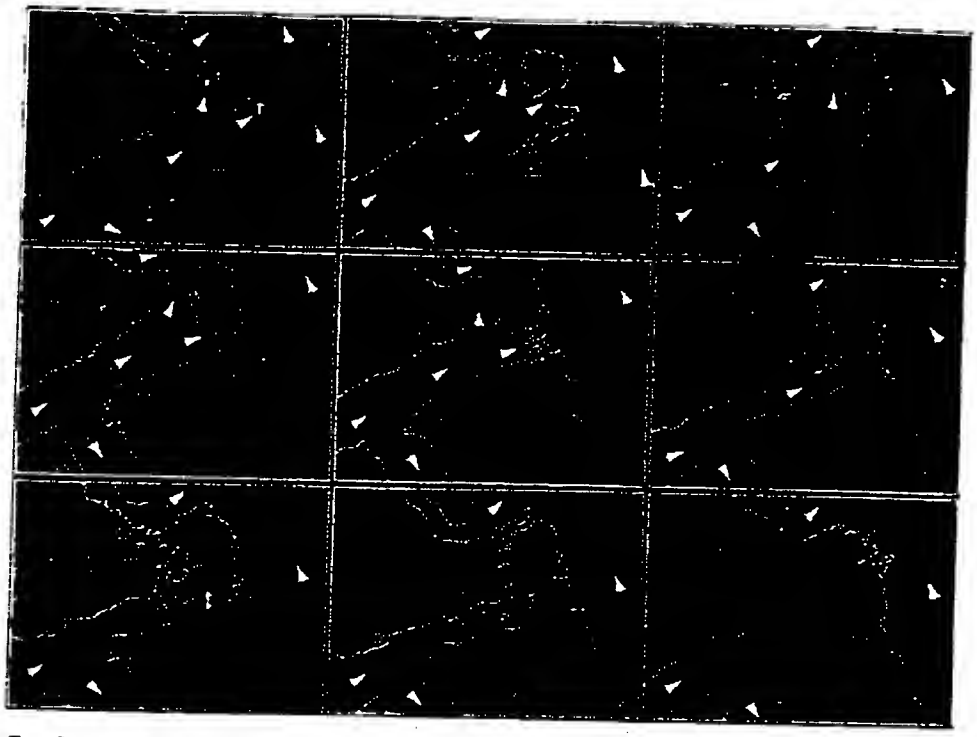


FIG. 5. A small tissue island located between a vascular branching point on Day 35. (A) From the left lower margin of the picture a vessel (average diameter 20 μm) connects to a larger vessel at right through two branches (*) and (†). (B) After a few minutes the tissue island has grown toward the lumen of branch (†) decreasing outflow into the large vessel. Additionally a tissue fold (Δ) projects into the lumen of branch (†) from the opposite wall. In C the fold has elongated and joined the tissue island to form an intervascular wall. The lumen of branch (†) is consequently closed and blood flows only through branch (*). In D the recently formed obstruction has opened up again by splitting into an ITS that allows for passage of flow through two channels. (E) A few minutes later the first ITS, located within branch (†) has split into two pillars. (F) During further development the two pillars formed in branch (†) again enlarge, resulting in occlusion of its lumen. In branch (*) three folds are now projecting from the island into the lumen, but the branch is still perfused. In G the upper fold connects with the tissue island. The lower two folds split into ITSs located completely inside the lumen (‡), while a new pillar has separated from the left wall of the branch. In H another new pillar can be detected at the terminal point, while the upper ITS has been incorporated into the occluding area of branch (*). Flow in this vessel is stagnant as parts of both branches are occluded now. In I the final stage of this sequence is reached as flow is diverted from the main stem into another branch located at the lower margin of the picture and the original connection with the large vessel at right is no longer visible.

a dense capillary network consisting of areas with fields of tissue pillars or in vascular occlusion that could cause regression. New formation of vascular segments by splitting of existing ITSs and their occlusion was subsequently detected next to each other (pillars formed by splitting, 0.8/hr; pillars formed by folding of the vascular wall, 1.25/hr; fusion of pillars or ITSs and connection of intraluminal folds with the opposite capillary wall, 1.5/hr; sprouts, 0.7/hr). Figure 5 demonstrates the occlusion of two vascular branches due to formation of intraluminally projecting tissue folds and their connection to the opposite vascular wall (Figs. 5A–5C). Each of these folds subsequently formed a very tiny intervascular wall. In the following stage, these walls split into ITSs and later into tissue pillars, and blood flowed around them (Figs. 5D and

5E). Here, the formation of numerous tissue pillars did not lead to a dense capillary area as could be observed in other cases. However, growth of pillars. ITSs, and the tissue island from which they were derived narrowed the lumen of the branches (Figs. 5F–5H). It finally resulted in connection of these structures and complete occlusion of both vessels (Fig. 5I).

During Week 6 the dense capillary areas underwent rapid remodeling and expansion, resulting in a peak in pillar formation by splitting of ITSs (7.6/hr).

Generally sprouts and tissue pillars could be observed throughout all 6 weeks of investigation. Within an observation period of 50 hr, as quantified from the length of the videotapes, we detected the formation of 53 sprouts and 169 pillars in the 40 tumors investigated.

DISCUSSION

We observed two different patterns of vascular network formation by analysis of the videotapes. The pattern of Weeks 1 and 2 was characterized by expansion of the vascular area into the nonvascularized one caused by IMG and sprout formation, while Week 4 exhibited a maximum of sprouting combined with splitting of ITSs. In these phases IMG and sprouting were thus observed frequently. After Week 4 sprouting decreased markedly and the patterns were dominated by remodeling caused by IMG which implicates a functional adaptation after a period of growth. Thus signs of remodeling were prominent in the later phases, in Week 3, and especially at the beginning of Week 5 until the end of Week 6, when they also included vascular occlusion. Our data suggest, however, an overlap of these phases. We have therefore focused on the most prominent patterns detected in each period.

The rapid vasodilatation mainly observed during the first 2 weeks of angiogenesis [11, 13–16] resulted in formation of "blood lakes." However, at some points the lumen did not expand along with the rest of the vessel, leading to formation of vertical tissue folds that project intraluminally and sometimes of sprouts located in the indentations between the folds. Further retraction of endothelial cells into the nonvascularized space finally led to thinning of these cells at the tip of the folds and sudden separation of the tips (as demonstrated in Figs. 1A and 2). The latter mechanism caused formation of free intraluminal tissue pillars or small ITSs and represents one mode of implementation of intussusceptive growth [10]. Morphological investigation of various stages of this type of pillar formation in the CAM demonstrated that the pillar constitutes a preformed structure that might be formed hours or even days before actual separation [10]. Since detachment of the pillar represents changes of cellular morphology at an extremely small scale, such as thinning of the endothelial cell extensions and cell membrane fusion with formation of a transcellular hole within an endothelial cell, it can occur suddenly (Fig. 1A). The latter process is comparable to endocytosis. Formation of transcellular holes also occurs when two sprouts connect. It may be hypothesized that the pillar constitutes the consequence of the sprout as it allows for expansion of the vascular area at a much smaller scale. However, the characteristic morphological composition of the tissue fold in the CAM suggests an active and principal role of the interstitial tissue organization that finally allows for endothelial cell migration and also for separation of tissue pillars [10]. The importance of the composition of the interstitium upon angiogenesis has been also demonstrated by analysis of the influence of gangliosides located in the extracellular matrix [17].

Investigation of vascular casts of varying rat organs revealed the existence of pillars within dilated vascular segments [8]. It seems plausible that these pillars correspond to those formed by folding of the vessel wall, suggesting that pillars constitute columns left behind while tissue is retracting. They subsequently stabilize dilated vascular segments and allow for further retraction [10].

Signs of vascular remodeling could be observed more frequently after the second week of network development. They were either represented by the flow-induced pillar or the splitting of large ITSs and intervascular walls. The flow-induced pillars detected frequently at the end of the second and during the third week are formed by connection of minute tissue folds projecting into the vascular lumen from opposite sides of the vascular wall (see also Fig. 1B). Investigation of capillary growth in the chicken CAM indicates that these folds are modeled by flow and their formation seems to be influenced by shear stress. Figure 3 demonstrates their unstable character in the tumor vasculature. Alterations of the circulation such as diminished flow from one direction can suddenly remove such a fold unless it is connected to the opposite wall to form a stable pillar. Analysis of vascular casts of various rat organs revealed the existence of pillars at triple branching points of the circulation [8], a location that corresponds to the type of pillar in Figs. 3 and 4. It is known that shear stress causes changes of morphology, growth status, and production and secretion of various effector molecules in cultured endothelial cells [18, 19]. Recently, it has been demonstrated that two angiogenic molecules, tPA and TGF-β1 are elevated after application of shear stress *in vitro* [20, 21]. It can thus be concluded that changes of intravascular shear stress might induce a cascade of physiological reactions in endothelial cells, and angiogenesis by tissue pillar formation could be one of them.

Vascular remodeling implemented by splitting of intervascular walls which results in fusion of neighboring vessels (Fig. 4) reached its maximum during Week 6. Vascular casts of various rat organs demonstrate the existence of pillars at quadruple branching points of the circulation [8], which suggests that they possibly resulted from splitting of intervascular walls. The formation of smaller vascular units in a sequential manner or, as interpreted by intussusception, the splitting of large tissue islands in ITSs and pillars of smaller size in each generation provides morphological support for the newly proposed fractal-based growth models [22].

During later stages of vascular network development the splitting of the tissue islands into ITSs and pillars can be fragmental as demonstrated in Fig. 5 and, in this case, it does not represent ongoing new formation of vascular segments. The tissue folds formed during this phase are thus not the result of retraction of vascular wall elements into the extravascular area, but they are active projections into the vessel lumen. This situation is the opposite of that observed during the first 2 weeks of angiogenesis (Fig. 2). The predominance of growth or regression thus seems to depend on the balance between vascular and extravascular space expansion. As many vascular segments are alternately opened and closed due to growth and occlusion, the pattern of blood flow is changed continuously. It has been demonstrated that regional tumor blood flow may vary on a time scale of minutes [23–27]. This phenomenon is termed intermittent blood flow and is still lacking a plausible explanation [14]. We suggest it could result from architectural changes caused by IMG.

Several modes of implementation of IMG, similar to those described in the present study, have been observed previously in the chicken CAM where the average time for observation of one event was 3–6 hr [10], compared to often less than 1 hr in the

tumor. This could be explained by the fact that tumor cells are involved in pillar formation which might account for altered behavior of these structures such as faster growth and rearrangement.

Vascular collapse can be proposed as an alternative explanation for the *in vivo* observed appearance of structures inside the lumen (Fig. 1B). These collapsed areas might open up and then disappear again or they might give rise to stable pillars corresponding to a mechanism of intussusception recently described in the rat lung [7]. Here, the pillar will form by connection of opposite vessel walls (Fig. 1B). The area of contact will be sealed off by formation of interendothelial junctions. After cell membrane fusion in the zone of contact, interstitial tissue will invade the pillar to form its core and stabilize its structure. This mechanism is similar to the one by which the flow pillar is formed at branching points of the circulation or in their vicinity: two tissue folds project intraluminally and connect (see Figs. 1B, 3 and 4).

On the other hand, pillars and ITSs are very small structures. It is thus hard to explain how vascular collapse might occur within a circumscribed area of about 1–2 μm and to account for the balance of forces that keeps the same lumen open within a space of similar size next to it. Finally, the existence of the different types of tissue pillars described here has been confirmed by analysis of light microscopic serial sections of the same tumor. They correspond to those observed in the chicken CAM and to the pattern of alterations observed by *in vivo* video microscopy in this organ supplemented by analysis of electron microscopic serial sections [10]. A thorough morphological investigation of serial sections at the ultrastructural level will enhance our understanding of modes of intussusception inside various tumors.

Taken together these results present the first demonstration of IMG in solid tumors. They also show for the first time *in vivo* detailed mechanisms of vascular network growth and remodeling during tumor angiogenesis. Knowledge about these mechanisms and their cellular implications constitutes the base for understanding the effects of angiogenic factors and their inhibitors and might enhance future therapeutic regimens.

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